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The goal of the present project is to define the mechanisms of neurotoxic interactions following exposure to pyridostigmine bromide (PB, 0.13, 1.3,13 mg/gk in water, oral), DEET (4.0, 40.0, 400.0 mg/kg in ethanol, dermal), and permethrin (0.013, 0.13, 1.3 mg/kg in ethanol, dermal) in male Sprauge-Dawley rats. The results are summarized below.

1. Combined exposure to the three test compounds for 28 days, alone or with stress produced neuropathological alterations in the brain and hepatic lesions.
2. The same treatments increased the permeability of the blood brain barrier (BBB), as assessed by [^{3}H]hexamethonium iodide uptake and horseradish peroxidase (HRP) staining.
3. Specific brain region exhibited decreased activity of AChE and ligand binding of m2 muscarinic acetylcholine receptors.
4. Exposure to DEET alone, or with permethrin significantly increased urinary excretion of 8-hydroxy-2'-deoxyguanosine.
5. Within 30 minutes of dermal application of 400 mg/kg DEET alone, or in combination with 1.3 mg/kg permethrin, 55% DEET and 38% permethrin was absorbed. Distribution of permethrin in tissues was slower than DEET. Combined application to both chemicals increased AUC for DEET but did not affect AUC_{plasma} of permethrin.

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Introduction

A) The Nature of the Problem

The goal of this project is to evaluate the possible interaction between DEET, permethrin and pyridostigmine bromide (PB) and the biological and pathological consequences of such interactions. Our standing hypothesis is that combined exposure to a mixture of chemicals would have enhanced, and in some cases deterimental toxicological effects than exposure with single chemical.

B) Approach

We have been testing the stated hypothesis that interactions between combined chemical exposure would result in greater toxicological and pathological changes, and certain environmental modifying factors such as stress would modulate the toxic effects in combined exposure scenario. In the previous year we have carried out dose-response studies ranging from 0.1-10 x the estimated human exposure on DEET, permethrin and PB. In those studies we carried out neurobehavioral as well as neurochemical assessment following exposure to a single or multiple chemicals **(See the manuscripts in Appendix)**. In the current studies, we have focused on the pathological consequences of sub-chronic exposure to single chemical or combined concurrent exposure. Additionally, we studied the effect of one environmental modifier, stress on the neurotoxicity associated with concurrent exposure to PB, DEET and permethrin. The pathological changes were studied by immunohistochemical evaluation of microtubule-associated protein-2 (MAP-2), glialfibrillary acidic protein (GFAP), and microglial activation by lectin binding. Furthermore, we evaluated the possible mechanism(s) of neurotoxic effects of single or combined exposure by assessing the permeability of blood-brain

barrier (BBB) and evaluating 8-hydroxy-2'-deoxyguanosine by HPLC. In the current studies, we also established the pharmacokinetic interaction between DEET and permethrin.

Body

CNS cyto-arhitecture is maintained by a complex cellular milieu that involves neurons and a variety of cells of astrocytic and glial origin. In order for the CNS to function properly and to respond to external stimuli, it is absolutely required that a proper communication is maintained within these cells. A major determinant of neuronal morphology is the cytoskeleton. Different components of cytoskeleton within the neurons and astrocytes provide forces to maintain the appropriate cellular structure, e.g. neuronal dendrites and axons are maintained in stable conditions by the force provided by the elements of cytoskeleton (Gavin, 1997). Such interactions are essential for proper synapse formation. The components of cytoskeleton are microfilament, intermediate filaments and microtubules. An important neuronal component, MAP-2 is enriched in dendrites and cell bodies (Tucker et al., 1988), in which it stabilizes the polymerized tubulin. Abnormal regulation of expression of MAP-2 causes suppression of neurite outgrowth and reduction in number of neurites in cultured neurons (Caceres et al., 1992). Similarly, aberrant intermediate filament proteins have been linked to diseases of neurodegeneration (Eliasson et al., 1999). A major component of astroytic intermediate filament, GFAP is upregulated in response to reactive gliosis as a consequence of a variety of insults, such as exposure to neurotoxic chemicals, trauma, and neurodegenerative diseases that affect the CNS (Eng and Ghirnikar, 1994). The function of GFAP is not well understood, but it has been suggested to play important role in long

term maintenance of brain cyto-architecture (Liedtke et al., 1996), proper function of BBB (Penky et al., 1998), and modulation of neuronal functions (Shibuki et al., 1996)

Microglia are involved in brain function under both normal and pathological conditions (Dickson et al, 1993, McGeer et al, 1993). In normal brain, resident ramified/resting microglia are activated to become rod-shaped or amoeboid shape in response to injury or toxic insult. Activated microglia proliferate, engulfing degenerating elements (Giulian et al, 1989, Stoll et al, 1989), while secreting cytotoxic agents that induce neuronal death and demyelination (Thery et al, 1991, Giulian et al, 1994).

Oxidative stress resulting from environmental toxicants has been considered as a cause of chemical exposure related diseases. In the CNS, oxidative DNA damage has been observed following ischemia (Cui et al., 2000). Increased formation of 8-hydroxy-2'-deoxyguanosine has been reported in response to exposure with environmental toxicants (Toraason et al., 1999, Halliwell, 1999). Thus, it is apparent that a diverse mechanism could be activated in response to toxic insult that may result in pathological changes.

Test Compounds:

In our studies, we chose to study PB, DEET, and permethrin because our previous results have demonstrated that a combination of these chemicals causes greater neurobehavioral and neurotoxic changes than each chemical alone (Abou-Donia et al., 2001a &b; Abou-Donia et al, 1996). In addition, thousands of U.S. Army personnel were presumably exposed to a combination of these chemicals during Persian Gulf War, and therefore, these studies may have human relevance.

Pyridostigmine Bromide (PB): PB is a quaternary dimethyl carbamate that has been used for the treatment of myasthenia gravis (Breyer-Pfaff et al., 1985). It was given to veterans for prophylactic protection to shield acetylcholinesterase (AChE) from the nerve agent poisoning by reversibly inhibiting 30-40% of the AChE in the peripheral nervous system, thus protecting the enzyme from irreversible inhibition by nerve agents (Blick *et al.,* 1991). The enzyme activity is restored following spontaneous decarbamylation resulting in near normal neuromuscular and autonomic functions (Blick, *et al.,* 1991). Toxic symptoms associated with PB overdose are primarily associated with over-stimulation of nicotinic and muscarinic receptors in the peripheral nervous system resulting in exaggerated cholinergic effects such as muscle fasciculations, cramps, weakness, muscle twitching, tremors, respiratory difficulty, gastero-intestinal tract disturbances and paralysis (Abou-Donia, 1994). The major metabolic product of PB is 3-hydroxy-*N*-methylpyridinium resulting from the carbamate hydrolysis that abolishes its cholinergic action (Kornfeld et al., 1970, 1971). Central nervous system effects of PB are not observed unless blood-brain barrier (BBB) permeability is compromised, because PB does not cross the BBB owing to the positive charge on the quaternary pyridinyl nitrogen (Birtley, *et al.,* 1966).

***N,N*-diethyl-*m*-toluamide (DEET):** DEET is commonly used as an insect repellant against mosquitoes, flies, ticks and other insects in the form of lotion, stick or spray (Robbins and Cherniack, 1986, McConnell *et al.,* 1986). Extensive and repeated topical application of DEET resulted in human poisoning including death (Gryboski *et al.,* 1961; Roland *et al.,* 1985; Edwards and Johnson, 1987). The symptoms associated with DEET poisoning are characterized by tremors, restlessness, difficulty in speech, seizures,

impairment of cognitive function and coma (McConnell *et al.,* 1986). Extremely high levels of DEET exposure has been reported to cause spongiform myelinopathy (Verschoyle *et al.,* 1992). Because DEET efficiently crosses the dermal barrier (Windheuser *et al.,* 1982; Hussain and Ritchel, 1988) and localizes to dermal fat deposits (Blomquist and Thorsell, 1977: Snodgrass *et al.*, 1982), it is possible that DEET may enhance the availability of drugs and toxicants in other organs and cause regulatory changes such as changes in blood brain permeability. However, it is not known with certainty that DEET could enhance the neurotoxicity associated with permethrim or PB because of its lipophillic nature.

Permethrin: Permethrin is a type I synthetic pyrethroid insecticide that exists in four different stereoisomers (Casida *et al.,* 1983). Its insecticidal activity persists for several weeks following a single application. Permethrin intoxication results as a consequence of modification of sodium channel such that it remains open for a longer time, leading to repetitive discharges after single stimulus (Narahashi, 1985). This repetitive nerve action is associated with tremors, hyperactivity, ataxia, convulsions, and in some cases to paralysis. It has been reported that high dose of permethrin given to rats induced peripheral nerve damage through its effects on sodium channels (Aldridge, 1990).

Key Research Accomplishments

During the preceding year we have carried out following investigations that fall within the objectives of the initial proposed work.

1. Neuropathological changes in the central nervous system (CNS) of rats following sub-chronic dermal exposure with 1x doses of DEET (40 mg/kg) and permethrin (0.13mg/kg), alone, and in combination.

In these studies, the animals were exposed with DEET and permethrin daily for 60 days and at the termination of the experiment gross histopathological evaluations of the CNS were carried out by H&E staining. Immunohistochemical assessment with anti glialfibrillary acidic protein (GFAP) and anti microtubule-associated protein (MAP-2) revealed extensive glial reactivity and neuronal dendritic loss.

2. Blood-Brain Barrier (BBB) permeability and cholinergic changes following exposure to stress with a combination of pyridostigmine bromide (PB), DEET, and permethrin.

In these studies, the animals were treated concurrently with PB (1.3 mg/kg, oral), DEET (40 mg/kg. dermal) and permethrin (0.13mg/kg, dermal), alone or in combination with stress for 28 days. The animals were stressed by placing them daily in Plexiglas restrainer for 5 minutes. At the termination, on day 29 the animals were evaluated for BBB permeability and biochemical evaluations for cholinergic system. The BBB permeability was assessed by the uptake of [^{3}H]hexamethonium iodide and crossing of horseradish peroxidase (HRP) into the CNS. CNS cholinergic system was studied by evaluating the acetylcholinesterase (AChE) activity and [^{3}H]AFDX-384 ligand binding for m2 muscarinic acetylcholine receptor in the brain regions. A significant increase in

the BBB permeability was observed following treatment with combination of chemicals and stress. Cerebral cortex, white matter, deep gray matter and brainstem from the animals co-exposed with chemicals and stress exhibited significant HRP staining as compared with chemicals or stress alone. Brain regions showed a significant inhibition in AChE activity and a decrease in m2 muscarinic acetylcholine receptor ligand binding densities.

3. Increased urinary excretion of 8-hydroxy-2'-deoxyguanosine following single dermal dose of DEET and permethrin, alone and in combination to rats.

In our efforts to monitor the exposure to DEET and permethrin, we evaluated the possibility of oxidative damage as a consequence of exposure to these chemicals alone or in combination. To this end, we monitored urinary excretion of 8-hydroxy-2'-deoxyguanosine by HPLC, as a bio-marker of oxidative DNA damage. The animals were exposed to single dose of DEET (400mg/kg, dermal in 70% ethanol) and permethrin (1.3 mg/kg, dermal in 70% ethanol), alone or in combination. Each animal was placed in metabolic cage and urine samples were collected at 2, 4, 8, 16, 24, 48, and 72 hrs after the treatment. Animals exposed to DEET alone or in combination with permethrin excreted a significantly higher levels of 8-hydroxy-2'-deoxyguanosine than controls. The levels of 8-hydroxy-2'-deoxyguanosine in the urine from the animals treated with permethrin alone were higher than the controls. However, these values were statistically not significant.

4. Pharmacokinetic interactions between a single dermal dose of DEET and permethrin.

In order to evaluate the pharmacokinetic interactions between DEET and permethrin, animals were exposed to single dermal dose of DEET (400mg/kg, in 70%

ethanol) and permethrin (1.3mg/kg in 70% ethanol), alone or in combination. Following treatment, the animals were maintained for 0.5, 1, 2, 4, 8, 24, 48 and 72 hrs, and urine samples were collected before sacrificing to collect plasma, liver, kidney, brain and testes. The samples were analyzed for DEET and permethrin and their metabolites by HPLC. Within 30 minutes of application, ~55% DEET and ~38% permethrin was absorbed, and by 72 hr almost all of DEET and ~96% permethrin was absorbed. DEET and its major metabolites, m-toluamide and m-toluic acid were detected in the plasma, following 1 hr of the treatment. Distribution of permethrin in the tissues was slower than DEET. Maximum plasma concentration of permethrin ~193ng/ml was detected after 24 hrs of treatment, whereas the corresponding values in the tissues ranged from ~52-109ng/g. m-phenoxybenzyl alcohol and m-phenoxybezoic acid were identified as permethrin metabolites in plasma, liver, and kidney 24 hrs after the treatment. One compartment pharmacokinetic modeling for DEET and permethrin in plasma suggested a terminal half-life of elimination from plasma of ~32.hr and ~22.9 hr for DEET and permethrin, respectively. Combined application of both chemicals increased the AUC_{plasm} of DEET as compared to the AUC_{plasma} of DEET following single treatment. There was no effect of DEET on the AUC_{plasma} of permethrin compared to treatment with permethrin alone.

Reportable Outcomes

Following are reportable outcomes from the studies conducted during the last year:

1. Neuropathological changes in the central nervous system (CNS) of rats following sub-chronic dermal exposure with 1x doses of DEET (40 mg/kg) and permethrin (0.13mg/kg), alone and in combination. These changes are characterized by an increase in immunohistochemical reactivity with anti glialfibrillary acidic protein (GFAP), suggestive of extensive glial reactivity and irregular dendritic staining with anti microtubule-associated protein (MAP-2) that suggest abnormal dendritic branching following treatment with a combination of DEET and permethrin.

2. Blood-Brain Barrier (BBB) permeability and cholinergic changes following exposure to stress with a combination of pyridostigmine bromide (PB), DEET, and permethrin. Concurrent treatment with PB (1.3 mg/kg, oral), DEET (40 mg/kg. dermal) and permethrin (0.13mg/kg, dermal), alone or in combination with stress for 28 days resulted in a significant increase in the BBB permeability following treatment with combination of chemicals and stress. Cerebral cortex, white matter, deep gray matter and brainstem from the animals co-exposed with chemicals and stress exhibited significant HRP staining as compared with chemicals or stress alone. Brain regions showed a significant inhibition in AChE activity and a decrease in m2 muscarinic acetylcholine receptor ligand binding densities following concurrent exposure with chemicals and stress.

3. Increased urinary excretion of 8-hydroxy-2'-deoxyguanosine following single dermal dose of DEET and permethrin, alone and in combination to rats. Exposure of rats to a single dose of DEET (400mg/kg, dermal in 70% ethanol) and permethrin (1.3 mg/kg,

dermal in 70% ethanol), alone or in combination resulted in significantly higher excretion of 8-hydroxy-2'-deoxyguanosine in the animals exposed to DEET alone or in combination with permethrin than controls.

4. Studies on pharmacokinetic interactions between a single dermal dose of DEET and permethrin. Following treatment of rats with a single dermal dose of DEET (400mg/kg, in 70% ethanol) and permethrin (1.3mg/kg in 70% ethanol), alone or in combination. HPLC analysis of DEET, permethrin and their metabolites showed that within 30 minutes of application, ~55% DEET and ~38% permethrin was absorbed, and by 72 hr almost all of DEET and ~96% permethrin was absorbed. DEET and its major metabolites, m-toluamide and m-toluic acid were detected in the plasma, 1 hr after the treatment. Distribution of permethrin in the tissues was slower than DEET. Maximum plasma concentration of permethrin ~193ng/ml was detected after 24 hrs of treatment, whereas the corresponding values in the tissues ranged from ~52-109ng/g. Permethrin metabolites, m-phenoxybenzyl alcohol and m-phenoxybezoic acid were identified in plasma, liver, and kidney 24 hrs after the treatment. One compartment pharmacokinetic modeling for DEET and permethrin in plasma suggested a terminal half-life of elimination from plasma of ~32.hr and ~22.9 hr for DEET and permethrin, respectively. Combined application of both chemicals increased the AUC_{plasm} of DEET as compared to the AUC_{plasma} of DEET following single treatment. There was no effect of DEET on the AUC_{plasma} of permethrin compared to treatment with permethrin alone.

In summary, these data suggest that toxic interactions between DEET, permethrin and PB and consequent health effects are dependent on exposure to other compounding factors such as stress and any other environmental conditions. It should be recognized

however, that health outcome in humans under PGW deployment conditions may have depended on the complex interactions between other factors that we have not studied.

Conclusions

The goal of the current project is to define the neurotoxic interactions between DEET, permethrin and Pyridostigmine bromide (PB) and molecular mechanisms of neuropathological effects resulting as a consequence of co-exposure to these chemicals. Rats were exposed to doses that included levels enough to be representative of human exposure. In our pathological experiments rats were exposed to 40 mg/kg DEET and 1.3 mg/kg permethrin in 70% ethanol by dermal exposure, and PB (1.3 mg/kg, in water oral) for varying length of time. Additionally, the animals were subjected to restraining stress by placing them in Plexiglas cylinders for five minutes daily. These doses and routes of exposure were chosen to closely approximate the possible mode of exposure to these chemicals during the Gulf War. In the studies to evaluate the pharmacokinetic interactions between DEET and permethrin, the doses chosen were higher than the physiological levels in order to maximize any interaction that would result from such exposure. Similar studies will be carried out using 0.1, and 1x doses of each chemical.

The animals were evaluated for the breakdown of blood-brain barrier (BBB) by [^{3}H]hexamethonium and horseradish peroxidase (HRP) uptake into the central nervous system. Gross histopathological evaluations of the CNS were carried out by H&E staining. Glial reactivity and neuronal dendritic loss were evaluated by immunohistochemical assessment with anti glialfibrillary acidic protein (GFAP) and anti microtubule-associated protein (MAP-2), respectively. CNS cholinergic system was studied by evaluating the acetylcholinesterase (AChE) activity and [^{3}H]AFDX-384 ligand binding for m2 muscarinic acetylcholine receptor in brain regions. Furthermore, we

evaluated the possibility of oxidative damage as a consequence of exposure to these chemicals, alone or in combination. To this end, we monitored urinary excretion of 8-hydroxy-2'-deoxyguanosine by HPLC, as a bio-marker of oxidative DNA damage. The pharmacokinetic interactions between DEET and permethrin was evaluated by treating the animals with a single dermal dose of DEET (400mg/kg, in 70% ethanol) and permethrin (1.3mg/kg in 70% ethanol), alone or in combination. Following treatment, the animals were maintained for 0.5, 1, 2, 4, 8, 24, 48 and 72 hrs, and urine samples were collected before sacrificing to collect plasma, liver, kidney, brain and testes. The samples were analyzed for DEET and permethrin and their metabolites by HPLC.

The data from these studies show that:

1. Daily dermal exposure to DEET and permethrin, alone or in combination for 60 days caused a significant cell damage as evaluated by H&E staining.

2. Daily dermal exposure to DEET and permethrin, alone or in combination for 60 days caused a significant increase in the expression of GFAP.

3 Following treatment with DEET and permethrin alone, and in combination for 60 days, MAP-2 expression was significantly decreased in cerebellum and cortex.

4. A significant increase in the BBB permeability was observed following concurrent treatment with DEET, permethrin and PB, and stress for 28 days as assessed by [3H]hexamethonium iodide uptake.

5. Cerebral cortex, white matter, deep gray matter and brainstem from the animals co-exposed with DEET, permethrin and PB and stress for 28 days exhibited significant HRP staining as compared with chemicals or stress alone.

6. Brain regions showed a significant inhibition in AChE activity and a decrease in m2 muscarinic acetylcholine receptor ligand binding densities following co-exposer with DEET, permethrin and PB and stress for 28 days.

7. There was a significantly higher levels of urinary excretion of 8-hydroxy-2'-deoxyguanosine in the animals exposed to DEET alone or in combination with permethrin urine than the controls.

8. Permethrin exposure alone caused an increase in the levels of 8-hydroxy-2'-deoxyguanosine in the urine, but these values were statistically not significant.

9. Following single dermal application of DEET (400mg/kg, in 70% ethanol) and permethrin (1.3mg/kg in 70% ethanol), alone or in combination, within 30 minutes of application, ~55% DEET and ~38% permethrin was absorbed, and by 72 hr almost all of DEET and ~96% permethrin was absorbed.

10. DEET and its major metabolites, m-toluamide and m-toluic acid were detected in the plasma, following 1 hr of the treatment.

11. Distribution of permethrin in the tissues was slower than DEET.

12. Maximum plasma concentration of permethrin ~193ng/ml was detected after 24 hrs of treatment, whereas the corresponding values in the tissues ranged from ~52-109ng/g. m-phenoxybenzyl alcohol and m-phenoxybezoic acid were identified as permethrin metabolites in plasma, liver, and kidney 24 hrs after the treatment.

13. One compartment pharmacokinetic modeling for DEET and permethrin in plasma suggested a terminal half-life of elimination from plasma of ~32.hr and ~22.9 hr for DEET and permethrin, respectively. Combined application of both chemicals increased the AUC_{plasma} of DEET as compared to the AUC_{plasma} of DEET following single

treatment. There was no effect of DEET on the AUC_{plasma} of permethrin compared to treatment with permethrin alone.

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Appendices

A. Manuscripts

Appendix 1: SUB-CHRONIC DERMAL APPLICATION OF *N,N*-DIETHYL *m*-TOLUAMIDE (DEET) AND PERMETHRIN TO ADULT RATS CAUSES DIFFUSE NEURONAL CELL DEATH AND CYTOSKELETAL ABNORMALITIES IN CEREBRAL CORTEX AND HIPPOCAMPUS, AND PURKINJE NEURON LOSS IN THE CEREBELLUM.
Appendix 2: STRESS AND COMBINED EXPOSURE TO LOW DAILY DOSES OF PYRIDOSTIGMINE BROMIDE, DEET, AND PERMETHRIN IN ADULT RATS LEADS TO BLOOD BRAIN BARRIER DISRUPTION AND NEUROCHEMICAL AND NEUROPATHOLOGICAL ALTERATIONS IN THE BRAIN.
Appendix 3: PHARMACOKINETS INTERATION BETWEEN DEET (*N,N*-DIETHYL *m*-TOLUMAMIDE) AND PERMETHRIN FOLLOWING DERMAL ADMINISTRATION IN RATS.

B. Published papers

Appendix 4: Abu-Quare A.W. and M.B. Abou-Donia, (2000). Simultaneous determination of pyridostigmine bromide, *N,N*-diethyl *m*-toluamide, permethrin, and their metabolites in rat plasma and urine by high-performance liquid chromatography. J. Chromtog. 749: 171-178.
Appendix 5: Abu-Qare, A. and Abou-Donia, M.B. (2000). Increased 8-hydroxy-2'-deoxyguanosine, a biomarker of oxidative DNA damage in rat urine following a single dermal dose of DEET (*N,N*-diethyl *m*-toluamide), and permethrin, alone and in combination. Toxicol. Lett. 117: 151-160.
Appendix 6: Abou-Donia, M.B., Goldstein, L.B., Jones, K.H., Abdel-Rehman, A., Damodaran, T.V., Dechkovskaia, A., Bullman, S., Amir, B.E.and Khan, W.A. (2001). Locomotor and sensorimotor performance deficit in rats following exposure to pyridostigmine bromide, DEET and permethrin, alone and in combination. Toxicol. Sci. 60: 305-314.
Appendix 7: Abou-Donia, M.B., Goldstein, L.B., Dechkovskaia, A., Bullman, S., Jones, K.H., Herrick, E.A., Abdel-Rehman, A. and Khan, W.A. (2001). Effects of daily dermal application of DEET and permethrin, alone and in combination on sensorimotor performance, blood-brain barrier and blood-testis barrier in rats. J. Toxicol. Environ. Health. 62: 523-541

C. Abstracts

Appendix 8:
a. Goldstein, L.B., Dechkovskaia, A., Bullman, S., Jones, K.H., Herrick, E.A., Abdel-Rehman, A, Khan, W.A. and Abou-Donia, M.B (2001). Daily dermal co-exposure of rats

to DEET and permethrin, produces sensorimotor deficit and changes in blood-brain barrier (BBB) and blood-testis barrier (BTB). The Toxicologist. 60: 59

b. Abu-Qare, A. and Abou-Donia, M.B. (2001). In vitro metabolism of pyridostigmine bromide (PB), DEET and permethrin, alone and in combination by human plasma and liver microsomes. The Toxicologist. 60: 89.

c. Abou-Donia, M.B, Khan, W.A., Suliman, H.B and Abdel-Rehman, A.A, (2001). Combined exposure to pyridostigmine bromide (PB), DEET and permethrin with stress increases blood-brain barrier (BBB) permeability and inhibits brain acetylcholinesterase in rats. The Toxicologist. 60: 238.

d. Abdel-Rehman, A.A, Suliman, H.B, Khan, W.A., and Abou-Donia, M.B. (2001). Apoptosis in testes induced by co-exposure of rats to DEET, permethrin and pyridostigmine bromide alone, and in combination with stress. The Toxicologist. 60: 282.

e. Amir, B.E, Abdel-Rehman, A.A., Goldstein, L.B., Dechkovskaia, A.M., Bullman, S. L., Khan, W.A and Abou-Donia, M.B. (2001). Exposure to pyridostigmine bromide (PB), DEET and permethrin, alone and in combination causes sensorimotor performance deficit and cholinergic alterations in rats. The Toxicologist. 60: 379.

Appendix 1
DAMD# 17-99-1-9020
Mohamed B. Abou-Donia

SUB-CHRONIC DERMAL APPLICATION OF *N,N*-DIETHYL *m*-TOLUAMIDE (DEET) AND PERMETHRIN TO ADULT RATS CAUSES DIFFUSE NEURONAL CELL DEATH AND CYTOSKELETAL ABNORMALITIES IN CEREBRAL CORTEX AND HIPPOCAMPUS, AND PURKINJE NEURON LOSS IN THE CEREBELLUM.

Running head: **Sub-Chronic application of *N,N*-Diethyl *m*-Toluamide (DEET) and Permethrin Causes Neuron Degeneration**

ABSTRACT: DEET and permethrin have been implicated as potential neurotoxic agents that may have played an important role in the development of illnesses in some veterans of the Persian Gulf War, as some service personnel used these chemicals during the war for their protection against insects . To determine the effect of sub-chronic dermal application of these chemicals on brain, we evaluated histopathological alterations in the brain of adult male rats following a daily dermal dose of DEET (40 mg/kg in 70% ethanol) or permethrin (0.13 mg/kg in 70% ethanol) or a combination of the two for 60 days. Control rats received a daily dermal dose of 70% ethanol for 60 days. Animals were perfused and brains were processed for morphological and histopathological analyses following the above regimen. In animals receiving either DEET or Permethrin, degenerating (eosinophilic) neurons were diffusely observed in distinct regions of the motor and somatosensory cortex, the hippocampus, and the cerebellum. In contrast, dying neurons were infrequent in animals receiving combined DEET and Permethrin. However, the density of surviving neurons in cerebral cortex, hippocampus and cerebellar Purkinje cell layer of these animals was significantly less than control animals, suggesting that in animals receiving combined DEET and permethrin neuronal cell death occurs earlier than animals receiving either DEET or permethrin alone. Analysis of glial fibrillary acidic protein immunoreactivity revealed significant hypertrophy of astrocytes in all three treated groups with maximal changes in rats receiving both DEET and permethrin. Further, surviving neurons in the latter group exhibited abnormal dendrites, characterized by wavy and beaded appearance with microtubule associated protein-2 immunostaining. Thus, sub-chronic dermal application of DEET and permethrin either alone or in combination leads to diffuse neuronal cell death in cerebral cortex, hippocampus and cerebellum of the adult brain. Collectively, the above alterations can lead to many physiological, pharmacological and behavioral abnormalities, particularly motor and sensory deficits and learning and memory dysfunction.

INTRODUCTION

During the Persian Gulf War (PGW), some service personnel were exposed to a variety of chemicals, including DEET and permethrin [18,2] that were used as a protection against insect-born disease. Some veterans have reported chronic symptoms including headache, loss of memory, fatigue, muscle and joint pain, and ataxia.

The insect repellent *N*, *N*-diethyl *m*-toluamide (DEET) and the pyrethroid insecticide, permethrin (3-phenoxybenzly [±]-cis, trans3- [2,2-dichlorovinyl]-2,dimethylcyclopropane-1-carboxylate) have been used extensively by humans since their introduction. DEET is used as an effective repellant against mosquitoes, flies, ticks and other insects in the form of lotion, stick or spray [26,21]. Extensive and repeated topical application of DEET can result in human and animal poisoning including death [15,27,10,20]. The symptoms associated with DEET poisoning are characterized by tremors, restlessness, difficulty with speech, seizures, impairment of cognitive function and coma [21]. High levels of DEET exposure have been reported to cause spongiform myelinopathy [35]. Because DEET efficiently crosses the dermal barrier [37,17,31] and localizes in dermal fat deposits [5,29], it is possible that DEET could enhance the availability of drugs and toxicants to other organs, including the brain [31].

Permethrin is a type I synthetic pyrethroid insecticide that exists in four different stereoisomers [8]. It provides insecticidal activity for several weeks following a single application. Permethrin toxicity is due to prolonged opening of the sodium channels, leading to repetitive discharges after a single stimulus [23]. This repetitive nerve action is associated with tremors, hyperactivity, ataxia, and convulsions and in some cases, paralysis.

The majority of the symptoms reported by the affected veterans of the PGW involve abnormal regulation of functions in either the central or peripheral nervous systems or both. Recent studies in our laboratory have suggested significant sensorimotor deficits and blood-brain barrier disruption following exposure to DEET and permethrin [1]. In this study, to determine the effect of sub-chronic dermal

application of these chemicals on brain at physiologically relevant doses, we evaluated histopathological alterations in the brain of adult male rats following a daily dermal application of DEET or permethrin or a combination of the two for 60 days. We demonstrate that sub-chronic application of DEET, permethrin, or the combination of DEET and permethrin leads to diffuse neuronal cell death in the cerebral cortex, the hippocampus and the cerebellum. In addition, surviving neurons in animals receiving combined DEET and permethrin exhibit considerable dendritic abnormalities suggestive of progressive degenerative changes, characterized by wavy and beaded appearance of microtubule-associated proteins. Taken together, the above changes can significantly compromise the functioning of the brain.

MATERIALS AND METHODS

Chemicals and antibodies

Technical-grade (93.6%) permethrin (+/-)-cis/trans-3- (2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester was obtained from Roussel Uelaf Corporation (Pasadena, TX). DEET (97.7%, *N,N*-diethyl *m*-toluamide) was purchased from Sigma Chemical Co., St. Louis, MO. The polyclonal antibody against glial fibrillary acidic protein (GFAP) was from Dako (Dako Labs, Carpinteria, CA) and the monoclonal antibody (SMI 52) against microtubule-associated protein-2 (MAP-2) was from Sternberger Monoclonals (Lutherville, MD). Avidin-biotin-complex (ABC) detection kits were purchased from Vector Labs (Burlington, CA). All other chemicals and reagents were of highest purity available from commercial sources.

Animals

Male Sprague-Dawley rats (200-250gms) obtained from Zivic Miller, Allison Park, PA were used. Animals were randomly assigned to control and treatment groups of five rats (n=5) and housed at 21-23^{0}C with 12-hr light/dark cycle. They were supplied with Purina Certified Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. The rats were allowed to adjust to their environment for a week before treatment. Animal care was in accordance with the Army Guidelines and Duke University Animals Care and Use Committee.

Dermal application of DEET and permethrin

DEET and permethrin, were applied on the skin of preclipped area 1^2 inch to give the desired concentration of test compounds in 1ml/kg of the vehicle solution. Groups of five rats received a daily dermal dose of 40 mg/kg DEET in 70% ethanol or 0.13 mg/kg permethrin in 70% ethanol, or the combination of DEET and permethrin. Control animals received an equal volume of the vehicle. The treatment was carried out daily, 7days a week, for 60 days. The doses of DEET and permethrin are based on an estimate of the exposure that may have occurred to army personnel during Gulf War (Abou -Donia, et al,2000). For combined exposure, each chemical was given at the single dose level.

Histopathological Assessment

One day after the last dose, animals in each group were anesthetized with pentobarbital (100 mg/kg) and perfused through the heart with saline followed by 4% paraformaldehyde and 0.1% gluteraldehyde in Tris buffer. The brains were removed, post-fixed, and embedded in paraffin. Three to four micrometer thick coronal sections were cut through different brain regions. In every brain, representative sections (n = 5) through the motor and sensory cortex, the septal hippocampus, and the cerebellum were processed and stained with hematoxylin and eosin (H&E) for light microscopy.

Microtubule associated protein-2 (MAP-2) and Glial fibrillary Acidic Protein (GFAP) Immunohistochemistry

Sections were deparaffinized and blocked with 10% normal serum (normal horse serum for MAP-2, and normal goat serum for GFAP) in 0.05M TBS for 30 minutes. Sections were incubated for overnight at room temperature in primary antisera diluted at 1:1000 for MAP-2 in 0.05M Tris buffer saline (TBS) containing 1% normal horse serum, and 1:10,000 for GFAP with 0.05M TBS containing 1% normal goat serum. Following a thorough rinse in 0.05% TBS, sections were incubated for 1 hr at room temperature in appropriate biotinylated secondary antibody (i. e., horse-anti-mouse IgG for MAP-2, and goat-anti-rabbit IgG for GFAP, diluted 1:200) containing 1% normal serum (horse serum for MAP-2 staining and goat serum for GFAP staining). Sections were rinsed with several changes of 0.05M TBS

and incubated for 1hr in the avidin-biotin peroxidase complex solution diluted 1: 25 in 0.05M TBS. Following this, the sections were rinsed with several changes of 0.05M TBS and incubated with 3,3-diaminobenzene tetrahydrochloride (DAB) for 10 min. The reaction was stopped by several rinses in 0.05M TBS. The sections were then dehydrated in alcohol, cleared in xylene and cover slipped with permount.

Quantitative evaluation of the number of surviving and dying neurons in different brain regions:

Numerical density of surviving and dying neurons per mm^2 area of tissue in H & E stained sections was measured for layers III and V of the motor cortex, granule cell layer of the dentate gyrus, pyramidal cell layer of CA1 and CA3 subfields of the hippocampus and Purkinje cell layer of the hippocampus. Five sections through each of the above brain regions were employed for these measurements in each animal belonging to the following four groups: (a) control animals (n = 5); (b) animals treated with DEET (n = 5); (c) animals treated with permethrin (n = 5); and (d) animals treated with both DEET and permethrin (n = 5). Measurements in sections from various groups were performed in a blinded fashion using experimental codes. The coding was such that animal treatments were not known during measuring; however, sections that came from the same animal were identified. All measurements were performed using a Nikon E600 microscope equipped with eyepiece grid. At a magnification of 400X (using 40X objective lens and 10X eyepieces), both surviving and dying neurons, within a unit area of each section (measuring 18,750 μm^2 layer III of cortex, 62,500 μm^2 for layer V of the cortex, 12,500 μm^2 for dentate granule cell layer, 6,250 μm^2 for CA1 pyramidal cell layer, 12,500 μm^2 for CA3 pyramidal cell layer, and 6,250 μm^2 for Purkinje cell layer of the cerebellum) were counted. For measurement of surviving neurons, only those which exhibited hematoxylin stained nucleus with a clear nucleolus were counted. For measurement of dying neurons, only those neurons that exhibited dense eosinophilic staining in both soma and proximal dendrites were counted. Finally, the density of neurons per unit area was transformed to the numerical density per mm^2 area of respective brain region.

Statistical Analyses

The mean value for each of the six brain regions (layers III and V of the motor cortex, granule cell layer of the dentate gyrus, CA1 and CA3 pyramidal cell layers of the hippocampus, and Purkinje cell layer of the cerebellum) was calculated separately for each animal by using data from 5 sections before the means and standard errors were determined for the total number of animals included per group. Mean values between different groups of animals were compared separately for each of the above brain regions using one-way ANOVA with Student's Newman-Keuls multiple comparison post-hoc test.

RESULTS

General Observations

The clinical condition of animals treated with daily dermal application of DEET, permethrin, or the combination of DEET and permethrin was not different from control animals. In addition, no differences were observed in the weights of animals between treated and control animals.

Histopathological Changes

Evaluation of brain sections stained with hematoxylin and eosin (H and E) clearly revealed neuronal degeneration in rats treated with DEET, permethrin, or the combination of DEET and permethrin, in comparison to vehicle treated rats. Degenerating neurons were characterized by eosinophilic staining of both cell body and proximal dendrites. In contrast, the healthy neurons in the same section exhibited hematoxylin stained nucleus (with a clear nucleolus) and eosin stained perinuclear cytoplasm. The brain regions where neuronal degeneration was most obvious include motor and sensory areas of the cerebral cortex, dentate gyrus, CA1 and CA3 subfields of the hippocampus, and cerebellar Purkinje cell layer. Other areas of the brain, though showed occasional dying (eosinophilic) neurons, the overall cytoarchitecture remained comparable to those in control (vehicle-treated) rats.

Alterations in the cerebral cortex

In animals treated with either DEET or permethrin alone, both superficial and deeper regions of motor and somatosensory cortex exhibited degenerating neurons in H and E stained sections. In superficial region (layers I-III; Fig. 1), degenerating neurons were conspicuous in layers II and III Majority of degenerating neurons in these layers were of pyramidal type with prominent eosinophilic apical dendrites (Fig. 1 [A2, A3]). The overall degree of neuron degeneration was comparable between animals treated with DEET and animals treated with permethrin. In deeper regions of the cortex (layers IV-VI), degenerating neurons were mostly observed in the layer V. These are larger pyramidal neurons with prominent apical and basal dendrites emanating from a larger pyramidal-shaped cell body (Fig. 2 [A2, A3]). The extent of degeneration appeared more with exposure to DEET than with exposure to

permethrin. Further, in addition to the presence of many degenerating neurons, both superficial and deeper regions of the cortex in animals treated with either DEET or permethrin exhibited clearly reduced packing density of surviving neurons, in comparison to the cortex of control animals (Figs. 1 [A1-A3] and 2 [A1-A3]. The adjacent sections stained for MAP-2 revealed significantly reduced MAP-2 positive dendrites in animals treated with either DEET or permethrin, in comparison to control animals (Figs 1 [B1-B3] and 2 [B1-B3]. The MAP-2 expression in dendrites also appeared somewhat disrupted and scarcer. Further, immunostaining with GFAP demonstrated increased GFAP expression in animals treated with either DEET or permethrin compared to control animals (Figs. 1 [C1-C3] and 2 [C1-C3]. Thus, the results of both MAP-2 and GFAP immunostained sections clearly corroborate the DEET and permethrin induced neurodegeneration, as observed in H and E stained samples.

In animals treated with both DEET and permethrin, degenerating (or eosinophilic) neurons were infrequent in both superficial and deeper regions of the cortex (Figs 1 [A4] and 2 [A4]). The packing density of surviving neurons, however, appeared less than that of control animals (Figs 1 [A1, A4] and 2 [A1, A4]). Areas devoid of neurons were conspicuous in layers II and V of the cortex (Figs. 1 [A4] and 2 [A4]). Thus, the lack of degenerating (eosinophilic) neurons in animals receiving both DEET and permethrin appeared to be due to early cell death of neurons following the combined exposure in comparison to animals receiving either DEET or permethrin, alone. The adjacent sections stained for MAP-2 and GFAP corroborated the above findings by exhibiting greatly reduced MAP-2 staining of dendrites, and significantly enhanced GFAP staining of astrocytes (Figs 1 [B4, C4] and 2 [B4, C4]. The GFAP positive astrocytes were clearly of reactive type and exhibited characteristic GFAP expression in their soma. In addition, the pattern of MAP-2 expression differed from both control animals and animals treated with either DEET or permethrin by showing a lack of expression in soma of neurons, and by their wavy and fragmented appearance in dendrites throughout the thickness of the cortex (Figs. 1 [B4] and 2 [B4]).

Quantification of surviving neurons per mm^2 area of layers III and V of the motor cortex revealed that animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibited a significant decrease in the number of surviving neurons in both layers III and V ($p < 0.01$; Fig. 3), in comparison to control animals. Analysis of dying neurons revealed that, in layer III of the motor cortex, animals treated with DEET exhibited a significant increase in the number of dying neurons, in comparison to control animals, animals treated with permethrin, and animals treated with both DEET and permethrin ($p < 0.01$; Fig. 3). In layer V of the motor cortex, animals treated with either DEET or permethrin exhibited a significant increase in dying neurons compared to control animals ($p < 0.001$ and $p < 0.01$ respectively; Fig. 3). Further analysis showed that exposure to DEET alone resulted in a significantly increased number of dying neurons than animals treated with permethrin alone ($p < 0.05$) and animals treated with both DEET and permethrin ($p < 0.001$; Fig. 3). Thus, a significant neuronal cell death occurs in motor cortex following sub-chronic dermal application of DEET and permethrin either, alone or in combination; however, the overall neuron loss in cortex is significantly greater with application of DEET alone, compared to exposure to permethrin alone and a combination of DEET and permethrin. This may suggest that the extent of DEET-induced neuron loss wanes when both DEET and permethrin were applied together. Nevertheless, astrocytic and neuronal cytoskeletal abnormalities were more prominent in animals treated with combined DEET and permethrin.

[Figures 1, 2, and 3 here]

Alterations in the hippocampal formation

Neuronal degeneration was obvious in the dentate gyrus, and CA1 and CA3 subfields of the hippocampal formation following exposure to DEET and permethrin either alone or in combination. In dentate gyrus of animals treated with DEET and permethrin, degenerating neurons were observed in both the granule cell layer and the dentate hilus (Fig. 4 [A2-A3]). In animals treated with both DEET and permethrin, the thickness and cell packing density of granule cell layer appeared reduced compared to both control animals and animals treated with either DEET or permethrin. The GFAP immunoreactivity was enhanced in molecular layer and hilus of all three treated groups, in comparison to control animals

(Fig. 4 [B1-B4]). The MAP-2 staining of the granule cell layer and the molecular layer did not show any significant differences between animals belonging to different treated groups but was clearly reduced compared to the control animals (data not shown). Thus, in the dentate gyrus, the overall damage was comparable following exposure to DEET and permethrin either alone or in combination.

In CA1 subfield of the hippocampus, degenerating neurons were clearly observed in the stratum pyramidale of animals treated with either DEET or permethrin alone, compared to control animals (Fig. 5 [A1-A3]). In animals treated with DEET and permethrin, the thickness and cell packing density of stratum pyramidale appeared significantly reduced compared to control animals but was comparable to animals treated with either DEET or permethrin (Fig. 5 [A4]). The MAP-2 staining of adjacent sections demonstrated a significantly reduced density of MAP-2 positive dendrites in animals belonging to all three treated groups compared to control animals (Fig. 5 [B1-=B4]). In animals treated with DEET, MAP-2 positive apical dendrites in stratum radiatum were thinner and appeared disrupted whereas, in animals treated with permethrin, MAP-2 positive dendrites appeared to be either beaded or arranged in aggregates with highly conspicuous vacant spaces between them. In animals treated with both DEET and permethrin, MAP-2 positive dendrites were wavy and thinner. The appearance of MAP-2 staining of apical dendrites in all treated groups highly contrasted with homogenous MAP-2 staining observed in control animals. Immunostaining of neighboring sections for GFAP demonstrated enhanced GFAP immunoreactivity in animals belonging to all three treated groups compared to control animals (Fig. 5 [C1-C4]). Thus, the hippocampal CA1 region exhibits neuronal cell death as well as dendritic abnormalities in surviving neurons. The overall damage appears similar following exposure to DEET, permethrin, or the combination of DEET and permethrin though the type of abnormality in MAP-2 expression varied between different treated groups.

In CA3 subfield of the hippocampus, degenerating neurons were clearly observed in the stratum pyramidale of animals treated with DEET alone, compared to control animals (Fig. 6 [A1-A2]). In animals treated with permethrin alone, degenerating neurons were not observed and the cell packing density appeared closed to that of control animals (Fig. 6 [A3]). In animals treated with combined

DEET and permethrin, the cell packing density of stratum pyramidale appeared reduced compared to both control animals and animals treated with permethrin alone but comparable to animals treated with DEET (Fig. 6 [A4]). The MAP-2 staining of adjacent sections demonstrated a reduced density of MAP-2 positive dendrites in animals belonging to all three treated groups compared to control animals (data not shown). The GFAP immunostaining of neighboring sections showed enhanced GFAP immunoreactivity in animals belonging to all three treated groups compared to control animals (Fig. 6 [C1-C4]). Thus, in hippocampal CA3 region, maximal damage occurred following exposure to either DEET alone or combined DEET and permethrin.

Quantification of surviving and dying neurons per mm^2 area of granule cell layer of the dentate gyrus and pyramidal cell layer of subfields CA1 and CA3 (Fig. 7) demonstrated the following. In dentate granule cell layer, animals treated with DEET, permethrin or a combination of DEET and permethrin exhibited a significant decrease in surviving neurons, in comparison to control animals ($p < 0.01$; Fig. 7). Further, animals treated with either DEET or permethrin exhibited a significant decrease in surviving neurons, in comparison to animals treated with combined DEET and permethrin ($p < 0.01$; Fig. 7). Analysis of dying neurons showed that only animals treated with either DEET or permethrin exhibited a significant number of dying neurons compared to control animals ($p < 0.05$; Fig. 7). Further, the number of dying neurons with exposure to DEET alone was greater than the number of dying neurons observed after exposure to combined DEET and permethrin ($p < 0.05$; Fig. 7). In CA1 subfield, only animals treated with either DEET or permethrin exhibited a significant decrease in the number of surviving neurons compared to control animals ($p < 0.05$; Fig. 7). Analysis of dying neurons also showed the same trend. Animals treated with either DEET or permethrin exhibited a greater number of dying neurons than both control animals ($p < 0.01$; Fig. 7) and animals treated with combined DEET and permethrin ($p < 0.05$; Fig. 7). In CA3 subfield, animals treated with DEET, permethrin or a combination of DEET and permethrin exhibited a significant decrease in surviving neurons, in comparison to control animals ($p < 0.01$; Fig. 7). Analysis of dying neurons revealed that animals treated with either DEET or permethrin exhibited a greater number of dying neurons compared to both control animals ($p < 0.01$) and animals

treated with combined DEET and permethrin ($p < 0.05$). Thus, a significant neuronal cell death occurs in the hippocampal formation following sub-chronic dermal application of DEET and permethrin either alone or in combination; however, the overall neuron loss in the hippocampal formation is significantly greater with application of DEET alone, compared to exposure to permethrin alone and a combination of DEET and permethrin.

[Figures 4, 5, 6, and 7 here]

Alterations in the cerebellum

In cerebellum, the most conspicuous damage following exposure to DEET or permethrin or combined DEET and permethrin was in the Purkinje cell layer. A large number of degenerating neurons were observed in animals treated with either DEET or permethrin compared to control animals (Fig. 8 [A1-A3]). In animals treated with combined DEET and permethrin, dying neurons were infrequent. However, the Purkinje cell density per length of Purkinje cell layer appeared reduced in comparison to both control animals (Fig. 8 [A4]. Wide areas of Purkinje cell layer lacking Purkinje neurons were frequently encountered in animals treated with both DEET and permethrin (Fig. 8 [A4]). Both thickness and cell packing density in granule cell layer appeared comparable between control animals and animals belonging to three treated groups. The GFAP immunostaining of neighboring sections showed a significantly enhanced GFAP immunoreactivity in the cerebellar white matter of animals belonging to the three treatment groups compared to control animals (Fig. 6 [B1-B4]). However, the maximal enhancement in GFAP immunoreactivity was observed in animals treated with both DEET and permethrin (Fig. 6 [B4]).

Quantitative analysis of Purkinje cells showed that animals treated with DEET, permethrin or a combination of DEET and permethrin exhibited a significant decrease in surviving neurons, in comparison to control animals ($p < 0.001$; Fig. 7). However, the degree of neuron loss with DEET exposure was greater than exposure to combined DEET and permethrin ($p < 0.05$; Fig. 7). Analysis of dying neurons revealed that animals treated with either DEET or permethrin exhibited a greater number of dying neurons compared to both control animals ($p < 0.05$; Fig. 7) and animals treated with combined

DEET and permethrin ($p < 0.05$; Fig. 7). Thus, as in the cerebral cortex and the hippocampus, a significant neuronal cell death occurs in the Purkinje cell layer of the cerebellum following sub-chronic dermal application of DEET and permethrin either alone or in combination; however, the overall neuron loss is significantly greater with application of DEET alone, compared to exposure to permethrin alone and a combination of DEET and permethrin.

[Figure 8 here]

DISCUSSION

The present study was designed to investigate the effects of daily dermal application of DEET and permethrin either, alone or in combination for 60 days on histopathological changes in the central nervous system of male rats. The route of exposure and dose levels of test compounds were chosen to closely reflect those present during the Gulf War [1]. The test-compounds were applied dermally at a dose of the estimated real life doses of 40 mg/kg/d for DEET and 0.13 mg/kg/d for permethrin. Our data suggest that exposure to physiologically relevant doses of DEET or permethrin or both DEET and permethrin for 60 days causes: (1) a diffuse neuronal cell death in the motor and somatosensory cortex, the hippocampal formation and the cerebellum; (2) a significantly atypical expression of cytoskeletal proteins within dendrites of surviving neurons of the cerebral cortex and the hippocampus; and (3) an increased expression of GFAP in astrocytes. The neuronal cell death was more pronounced in pyramidal neurons belonging to layers III and V of the motor cortex, pyramidal and granule neurons belonging to layers III-V of the somatosensory cortex, granule cells of the dentate gyrus, pyramidal neurons of hippocampal CA1 and CA3 subfields, and Purkinje cells of the cerebellum. The abnormalities in MAP-2 expression of surviving neurons were distinguished by a beaded, disrupted, or wavy appearance of MAP-2 positive elements within apical dendrites of cortical neurons and CA1 pyramidal neurons. The GFAP expression was exemplified by GFAP expression in soma of astrocytes and hypertrophy of astrocytic processes emanating from the soma.

The maximal neuronal loss was observed when animals were treated with DEET alone, compared to exposure to permethrin alone or a combination of DEET and permethrin. This may suggest that the extent of DEET-induced neuronal loss wanes when both DEET and permethrin were applied together. Nevertheless, the maximal alterations in the expression of MAP-2 and GFAP were observed when animals were treated with both DEET and permethrin. The above pattern of neurodegeneration and cytoskeletal alterations suggest that degenerative changes induced by co-exposure to DEET and permethrin are quite robust and detrimental to the normal functioning of the central nervous system, despite a reduction in the extent of overall neuronal cell loss. Reduced neuronal loss with exposure to

both DEET and permethrin likely reflects a decrease in effective concentration of chemicals at the neurotoxicity target, as concurrent exposure to chemicals can decrease their absorption.

In our previous studies in hens using sub-cutaneous route of exposure at relatively higher doses, we demonstrated that co-exposure to DEET and permethrin results in an enhanced level of toxicity than exposure to each chemical alone [2]. Our recent behavioral studies in rats showed that exposure to DEET or permethrin or both DEET and permethrin for 60 days using the same dose level used in the current study, leads to significant deficits in sensorimotor functions [1]. In addition, that previous study demonstrated subtle changes in the blood brain barrier following exposure to either DEET or both DEET and permethrin, and suggested that additional approaches such as histopathological evaluations may provide definitive proof of the changes in the CNS occurring as a consequence of DEET treatment alone or a combination with permethrin. Our present data provide clear histopathological evidence that sub-chronic exposure to DEET, permethrin, alone or in combination leads to significant neuronal cell death and cytoskeletal abnormalities in surviving neurons that could compromise functions of the brain.

The neurotoxic effects of DEET may be augmented, by both its increased localization into the CNS because of its lipophilicity, and because of decrease in the transport of otherwise critical molecules. Severe signs of CNS toxicity due to DEET and permethrin are apparent only at high doses, e.g., DEET induced signs of CNS depression, death and protracted seizure activity was observed at several dose levels in rats [36]. Similar complications have been observed in DEET poisoning in humans [25,21]. Symptoms such as daytime sleepiness and impaired cognitive functions have been shown to result from heavy DEET exposure, whereas gait balance and dexterity were moderately affected [21]. Additionally, lethargy has been noted as a prominent feature in severe acute DEET intoxication [32,30]. A relatively recent study found a decrease in motor activity in male and female rats after a single dose of DEET treatment [36]. Permethrin induced behavioral changes have also been documented in animals [16]. Permethrin-induced neurotoxic changes are characterized by aggressive sparring, increased sensitivity to external stimuli and fine tremors that progresses to whole-body tremors and prostration [34,35,4]. [22] reported a decrease in grip strength and induced head and forelimb shaking. Additionally, decreased

operant response rate, deficit in role mode performance and a decrease in turning-wheel activity has been observed [6,14]. Studies by Crofton and Reiter [9] have shown a decrease in locomotor activity in rats exposed to permethrin.

CNS cytoarchitecture is maintained by a complex cellular milieu that involves neurons and a variety of cells of glial origin. In order for the CNS to function properly and to respond to external stimuli, it is absolutely required that a proper communication is maintained within these cells. A major determinant of neuronal morphology is the cytoskeleton. Different components of cytoskeleton within the neurons and astrocytes provide forces to maintain the appropriate cellular structure, e.g., neuronal dendrites and axons are maintained in stable conditions by the force provided by the elements of cytoskeleton [13]. Such interactions are essential for proper synapse formation. The components of cytoskeleton are microfilament, intermediate filaments and microtubules. An important neuronal component, MAP-2 is enriched in dendrites and cell bodies [33], in which it stabilizes the polymerized tubulin. Abnormal regulation of expression of MAP-2 causes suppression of neurite outgrowth and reduction in number of neurites in cultured neurons [7]. Similarly, aberrant intermediate filament proteins have been linked to diseases of neurodegeneration [11]. Our data clearly show both decrease and abnormalities in MAP-2 expression following treatment with DEET or permethrin or both DEET and permethrin in the brain, particularly the cerebral cortex and the hippocampal formation. A decreased expression and beaded appearance of MAP-2 in dendrites would lead to destabilization of dendrites and can result in abnormal functioning of neurons particularly loss of synapses due to resorption of post-synaptic specializations such as dendritic spines. Such aberrant dendritic organization and the consequently altered connectivity in the cerebral cortex and the hippocampal formation could respectively have profound adverse influence on sensorimotor function and learning and memory.

A major component of astrocytic intermediate filament, GFAP is up-regulated in response to reactive gliosis as a consequence of a variety of insults, such as exposure to neurotoxic chemicals, trauma, and neurodegenerative diseases that affect the CNS [12]. The precise function of GFAP is not well understood, but it is believed to play an important role in the long-term maintenance of brain

cytoarchitecture [19], proper functioning of the blood brain barrier]24], and modulation of neuronal functions [28]. An increased expression of GFAP in the soma and processes of astrocytes in various brain regions exhibiting neuronal cell death indicates that neurodegenerative changes induced by exposure to DEET and permethrin either alone or in combination are quite robust and lead to a significant hypertrophy of astrocytes. This is because hypertrophied astrocytes (or reactive astrocytes) represent transformed resting astrocytes with increased GFAP accumulation, and this transformation occurs as a consequence of injury to the brain. The accumulation of reactive astrocytes can lead to increased generation of toxic mediators that may cause further pathological damages in the brain.

It should be noted that most of the earlier studies on the neurotoxic effects of DEET or permethrin used routes of exposure that are not directly germane to the contact exposure, as is believed to have occurred during the Gulf War. The results of this study, however, clearly suggest that sub-chronic dermal application of these chemicals leads to diffuse neuronal cell death and significant neuronal cytoskeletal abnormalities in many regions of the brain. The above alterations are likely the contributory factors for neurobehavioral abnormalities observed by others and us in adult rats following exposure to DEET and permethrin.

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FIGURE LEGENDS

Figure 1 - Alterations in the superficial layers (layers I-III) of the motor cortex following daily dermal application of DEET and permethrin. A1-A4, H&E staining; B1-B4, MAP-2 immunostaining; C1-C4, GFAP immunostaining. A1, B1, C1 are examples from a control rat. A2, B2, C2 are examples from a rat treated with DEET. A3, B3, C3 are examples from a rat treated with permethrin whereas A4, B4, C4 are examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in rats treated with either DEET or permethrin (arrows in A2 and A3). Whereas in the rat treated with both DEET and permethrin, vacant areas devoid of neurons (A4) and wavy appearance of dendrites (arrows in B4) are conspicuous. Note that, in all three treated groups, the overall MAP-2 immunoreactivity is significantly reduced (B2-B4) and GFAP immunoreactivity of astrocytes is significantly enhanced (arrowheads in C2-C4), in comparison to the control group (B1, C1). Scale bar, 100 μm.

Figure 2- Changes in the deeper layers (layers IV-V) of the motor cortex following daily application of DEET and permethrin. A1-A4, H&E staining; B1-B4, MAP-2 immunostaining; C1-C4, GFAP immunostaining. A1, B1, C1 are examples from a control rat. A2, B2, C2 are examples from a rat treated with DEET. A3, B3, C3 are examples from a rat treated with permethrin whereas A4, B4, C4 are examples from a rat treated with both DEET and permethrin. A large number of degenerating pyramidal neurons are clearly visible in layer V of the cortex in rats treated with either DEET or permethrin (arrows in A2 and A3). In the rat treated with both DEET and permethrin, vacant areas devoid of neurons (A4) and wavy appearance of dendrites (arrows in B4) are conspicuous. Note that, in all three treated groups, the overall MAP-2 immunoreactivity is significantly reduced (B2-B4) and GFAP immunoreactivity of astrocytes is significantly enhanced (arrows in C2-C4), in comparison to the control group (B1, C1). Scale bar, 100 μm.

Figure 3 – Histograms show the density of surviving (A) and dying (B) neurons/mm2 area of layers III and V of the motor cortex. Values represent means and standard errors (n = 5 per group). Analyses with one-way ANOVA shows significant differences for surviving neurons between groups ($p < 0.01$ in layer III, $p < 0.001$ in layer V). The post-hoc analysis with Student's Newman-Keuls multiple comparisons test further revealed that animals treated with DEET or permethrin alone exhibit a significant decrease in the number of surviving neurons, in comparison to control animals (layer III, $p < 0.05$; layer V, $p < 0.01$), Analysis of dying neurons revealed that, in layer III of the motor cortex, animals treated with DEET exhibit a significant increase in the number of dying neurons, in comparison to control animals, animals treated with permethrin, and animals treated with both DEET and permethrin ($p < 0.01$). In layer V of the motor cortex, animals treated with either DEET or permethrin exhibit a significant increase in dying neurons compared to control animals ($p < 0.001$ and $p < 0.01$ respectively). Further, exposure to DEET alone results in a significantly increased number of dying neurons than exposure to permethrin alone ($p < 0.05$) and exposure to both DEET and permethrin ($p < 0.001$).

Figure 4 – Alterations changes in the dentate gyrus following daily application of DEET and permethrin. A1-A4, H&E staining; B1-B4, GFAP immunostaining. A1, B1 are examples from a control rat. A2, B2 are examples from a rat treated with DEET. A3, B3 are examples from a rat treated with permethrin whereas A4, B4 are examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in the dentate granule cell layer (GCL) and the dentate hilus (DH) of rats treated with either DEET or permethrin (arrows in A2 and A3). In the rat treated with both DEET and permethrin (A4), both thickness and cell packing density of granule cell layer are reduced compared to the control rat. Note that GFAP immunoreactivity is up regulated in all three treated groups (B2, B3, B4). ML, molecular layer. Scale bar, 100 μm.

Figure 5 - Alterations in the CA1 subfield of the hippocampus following daily dermal application of DEET and permethrin. A1-A4, H&E staining; B1-B4, MAP-2 immunostaining; C1-C4, GFAP immunostaining. A1, B1, C1 are examples from a control rat. A2, B2, C2 are examples from a rat treated with DEET. A3, B3, C3 are examples from a rat treated with permethrin whereas A4, B4, C4 are

examples from a rat treated with both DEET and permethrin. A large number of degenerating pyramidal neurons are clearly visible in the stratum pyramidale (SP) of rats treated with either DEET or permethrin (arrows in A2 and A3). In the rat treated with both DEET and permethrin, both thickness and cell packing density of CA1 cell layer are reduced compared to the control rat (A4) and dendrites in stratum radiatum have wavy appearance (B4). Note that, in all three treated groups, the overall density of MAP-2 immunoreactive elements is significantly reduced (B2-B4) and the pattern of MAP-2 expression in dendrites is altered, in comparison to the control group (B1). Further, in all treatment groups (C2-C4), GFAP immunoreactive astrocytes are significantly increased in both stratum oriens (SO) and stratum radiatum (SR). Scale bar, 100 μm.

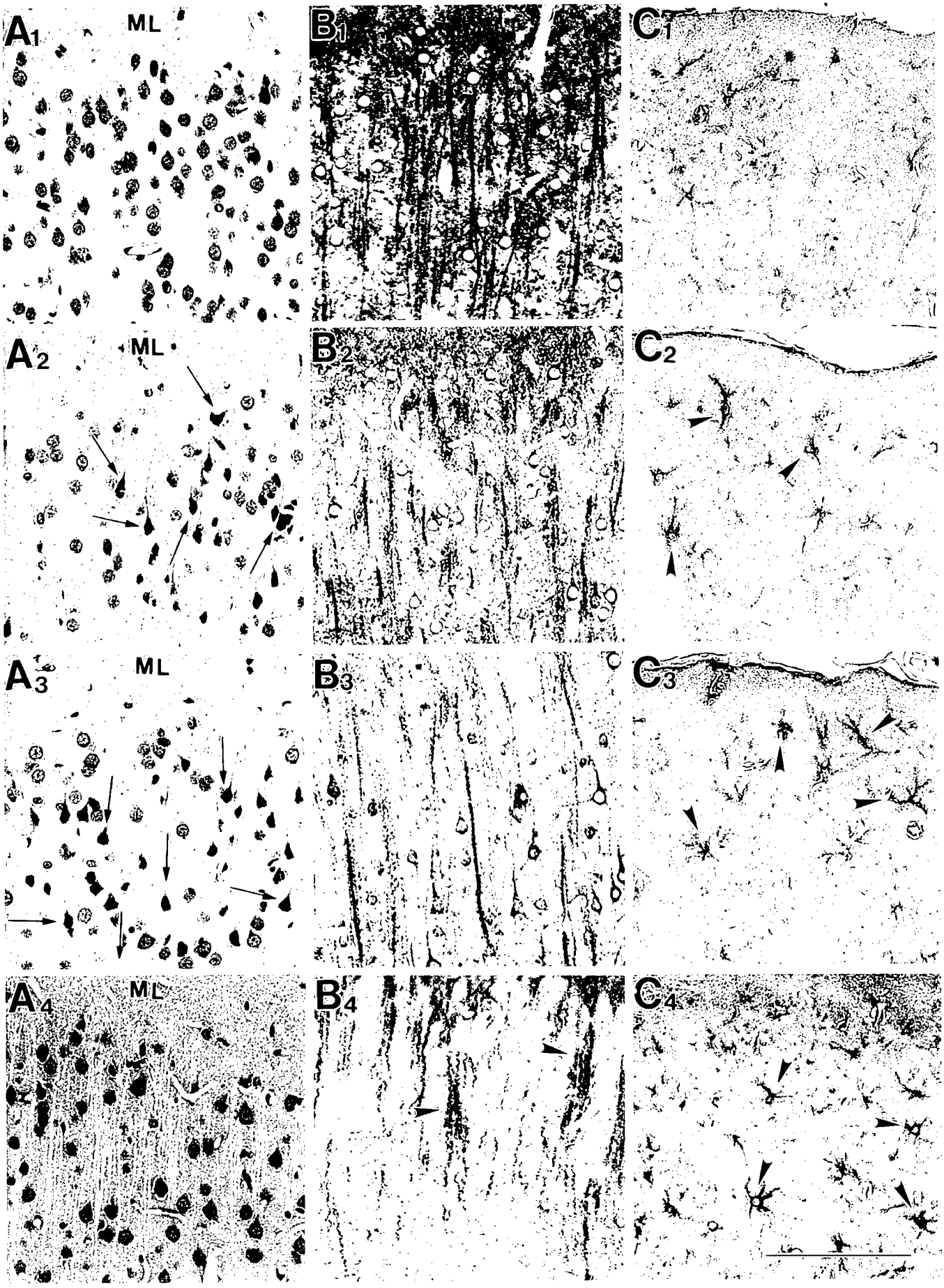
Figure 6 - Changes in the CA3 subfield of the hippocampus following daily application of DEET and permethrin. A1-A4, H&E staining; B1-B4, GFAP immunostaining. A1, B1 are examples from a control rat. A2, B2 are examples from a rat treated with DEET. A3, B3 are examples from a rat treated with permethrin whereas A4, B4 are examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in the stratum pyramidale (SP) of CA3 subfield of the rat treated with DEET alone (arrows in A2). In rats treated with permethrin alone (A3) and both DEET and permethrin (A4), the thickness and cell packing density of CA3 cell layer are reduced compared to the control rat. Note that GFAP immunoreactivity is up regulated in all three treated groups (B2, B3, B4). SO, stratum oriens; Scale bar, 100 μm.

Figure 7 – Histograms show the density of surviving (A) and dying (B) neurons per mm2 area of different cell layers of the hippocampal formation and Purkinje cell layer of the cerebellum. Values represent means and standard errors ($n = 5$ per group). Analyses with one-way ANOVA revealed significant differences between groups for both surviving neurons (dentate gyrus, $p < 0.001$; CA1 subfield, $p < 0.05$; CA3 subfield, $p < 0.001$; and cerebellum, $p < 0.0001$) and dying neurons (dentate gyrus, CA1 and CA3 subfields, $p < 0.01$; cerebellum, $p < 0.001$). The post-hoc analysis with Student's Newman-Keuls multiple comparisons test further revealed the following. In dentate granule cell layer, animals treated with DEET, permethrin or a combination of DEET and permethrin exhibit a significant

decrease in surviving neurons, in comparison to control animals ($p < 0.01$). Further, animals treated with either DEET or permethrin exhibit a significant decrease in surviving neurons, in comparison to animals treated with both DEET and permethrin ($p < 0.01$). Analysis of dying neurons (B) shows that animals treated with either DEET or permethrin exhibit a significant number of dying neurons compared to control animals ($p < 0.05$); the number of dying neurons with exposure to DEET alone is greater than with exposure to both DEET and permethrin ($p < 0.05$). In CA1 subfield, only animals treated with either DEET or permethrin exhibit a significant decrease in the number of surviving neurons compared to controls ($p < 0.05$). Analysis of dying neurons shows that animals treated with either DEET or permethrin exhibit a greater number of dying neurons than both controls ($p < 0.01$) and animals treated with both DEET and permethrin ($p < 0.05$). In CA3 subfield, animals treated with DEET, permethrin or a combination of DEET and permethrin exhibit a significant decrease in surviving neurons, in comparison to controls ($p < 0.01$). Analysis of dying neurons reveals that animals treated with either DEET or permethrin exhibit a greater number of dying neurons compared to both controls ($p < 0.01$) and animals treated with both DEET and permethrin ($p < 0.05$). In cerebellum, animals treated with DEET, permethrin or a combination of DEET and permethrin exhibit a significant decrease in surviving Purkinje cells, in comparison to control animals ($p < 0.001$). However, the degree of neuron loss with DEET exposure is greater than exposure to both DEET and permethrin ($p < 0.05$). Analysis of dying neurons showed that animals treated with either DEET or permethrin exhibit a greater number of dying neurons compared to both control animals and animals treated with both DEET and permethrin ($p < 0.05$).

Figure 8 – Alterations in the cerebellum following daily application of DEET and permethrin. A1-A4, H&E staining of the cerebellar cortex; B1-B4, GFAP immunostaining of the cerebellar white matter. A1, B1 are examples from a control rat. A2, B2 are examples from a rat treated with DEET. A3, B3 are examples from a rat treated with permethrin whereas A4, B4 are examples from a rat treated with both DEET and permethrin. A large number of degenerating Purkinje neurons are clearly visible in the Purkinje cell layer of rats treated with either DEET or permethrin (arrows in A2, A3). Arrows in A1-A4 point to surviving neurons. In rats treated with both DEET and permethrin (A4), a large area of Purkinje

cell layer is devoid of Purkinje neurons (bracketed area A4). Note that GFAP immunoreactivity in the white matter of the cerebellum is significantly up regulated in all three treated groups (B2, B3, B4) with maximal up-regulation in the group treated with both DEET and permethrin. GCL, granule cell layer; ML, molecular layer. Scale bar, 100 μm.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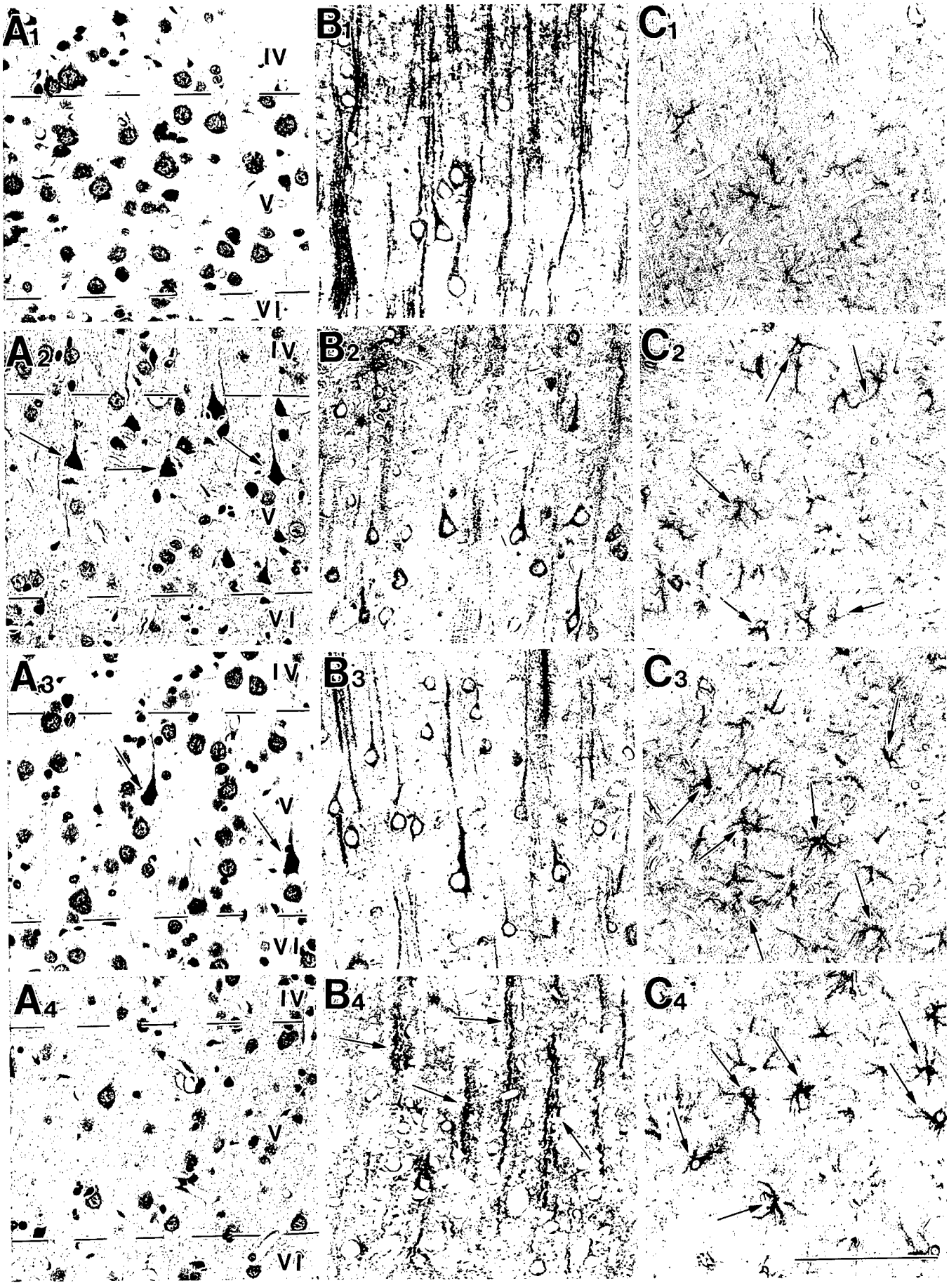
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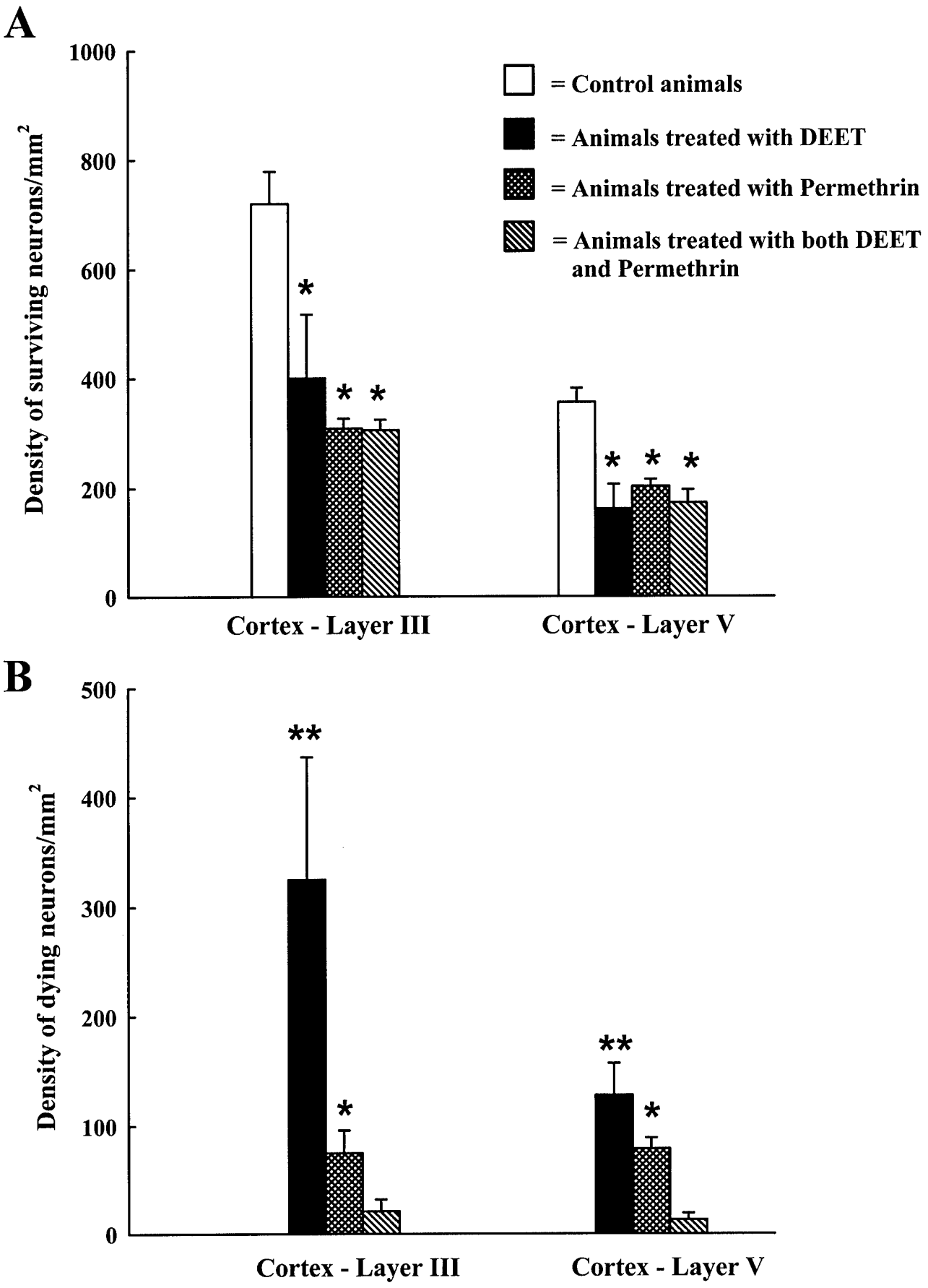
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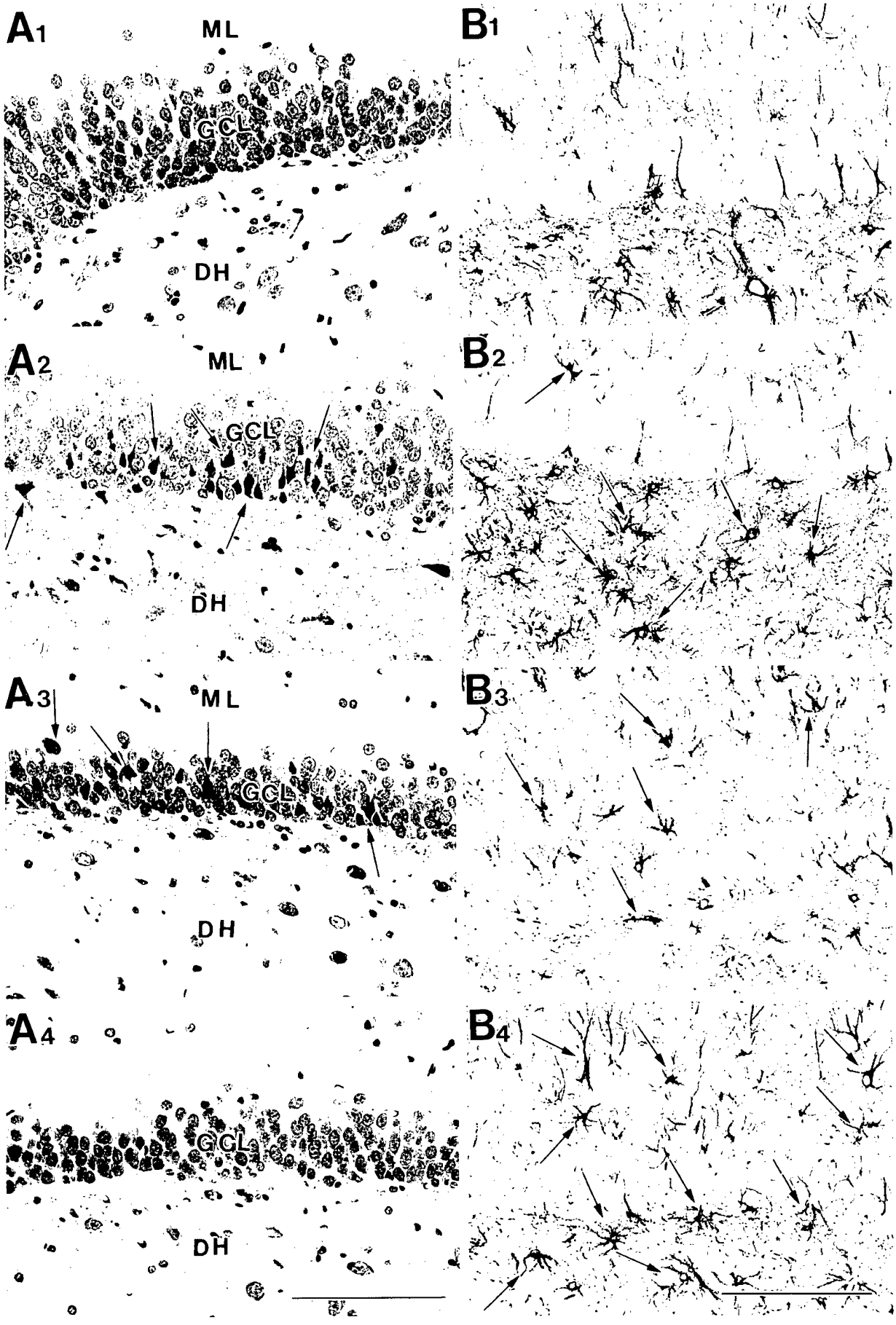
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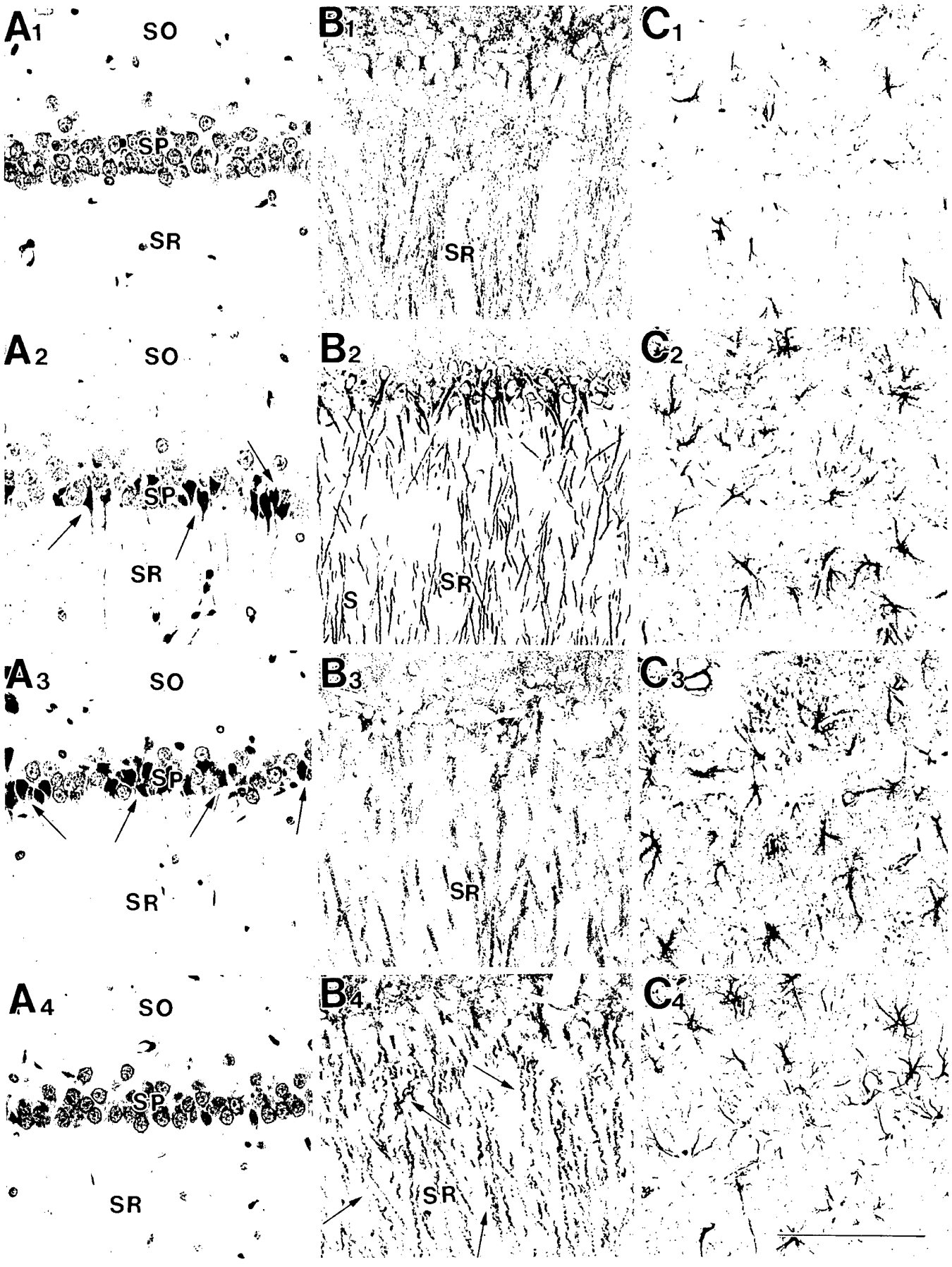
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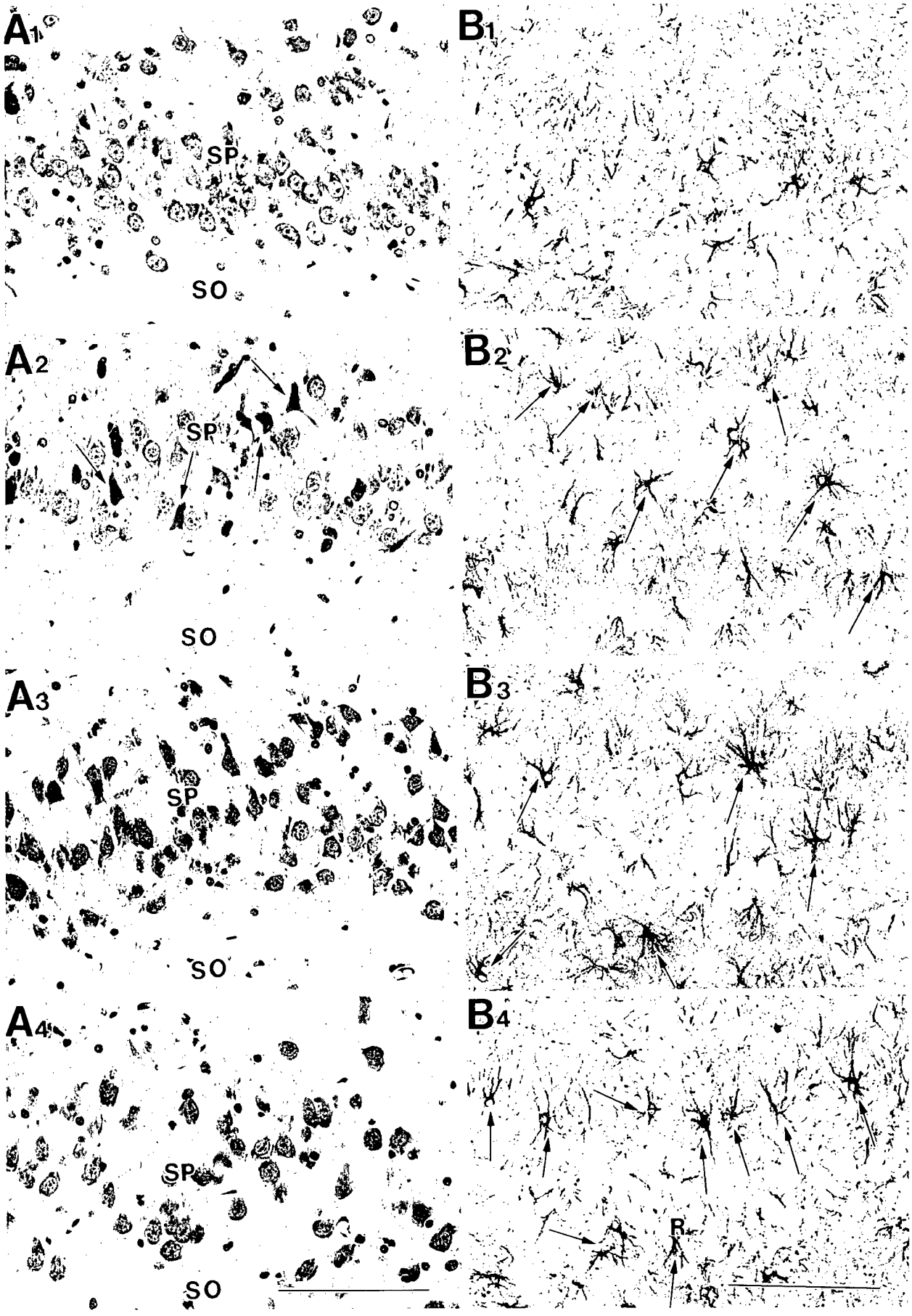
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B4
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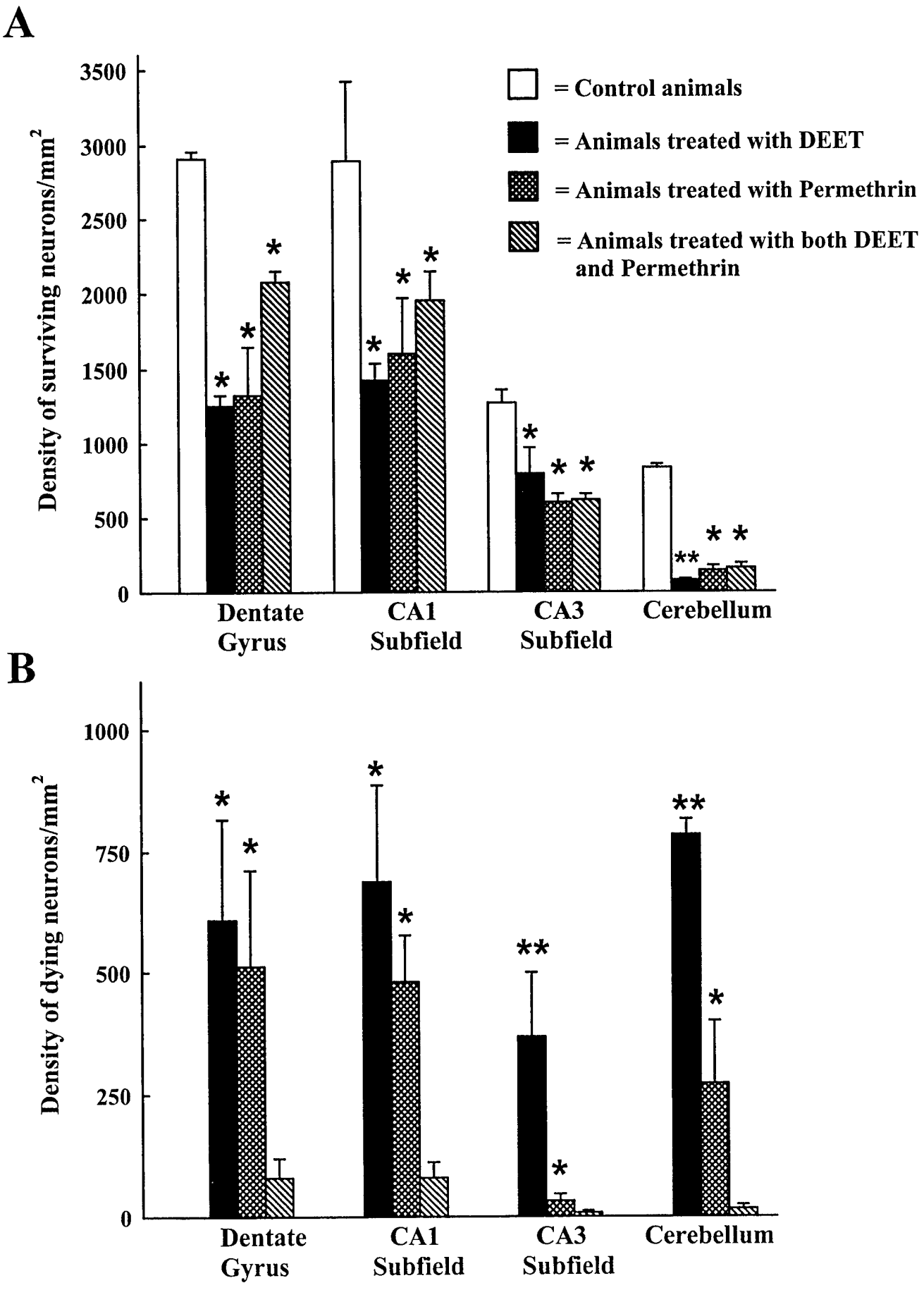
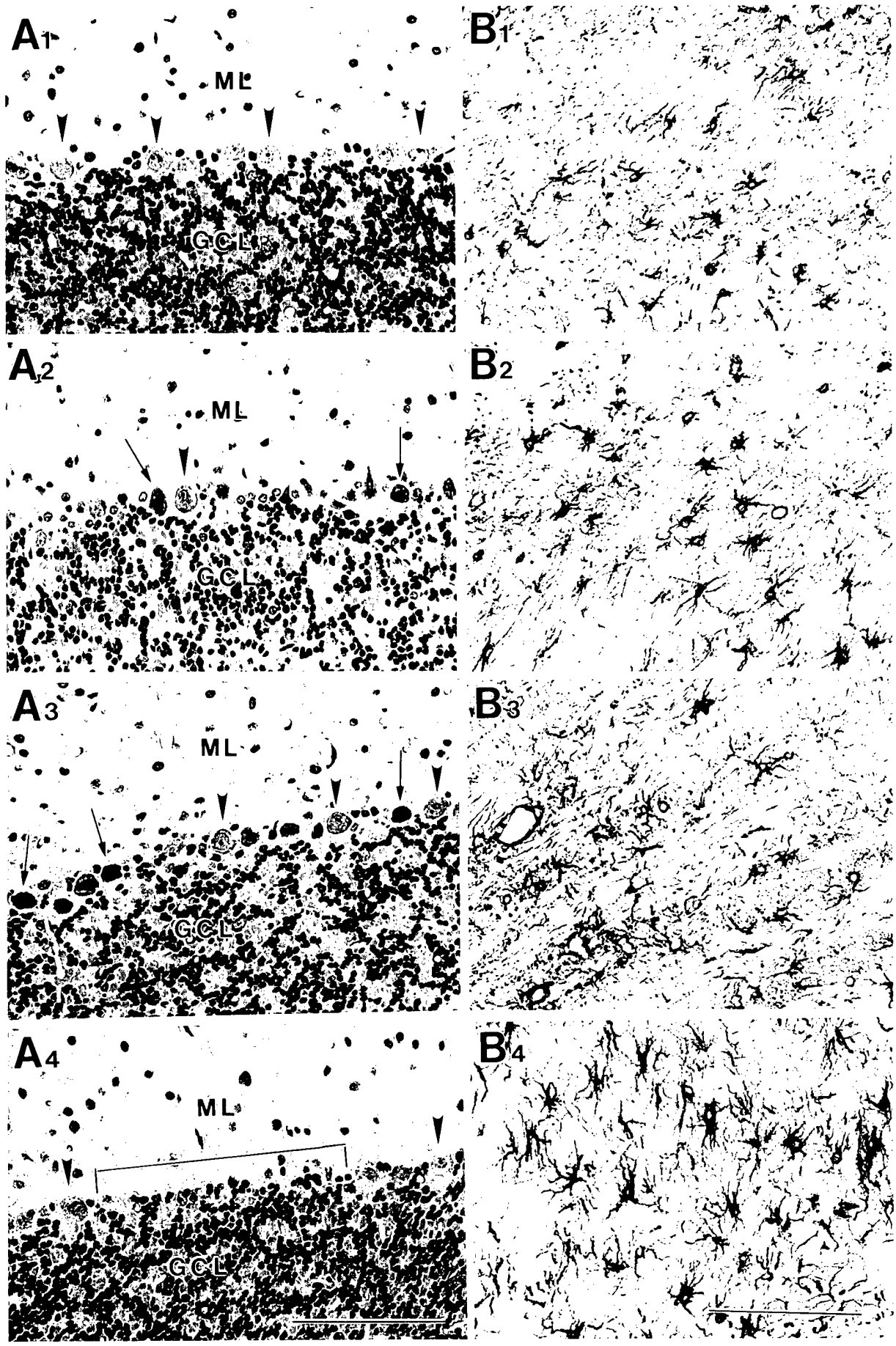

A
Density of surviving neurons/mm2
3500
3000
2500
2000
1500
1000
500
0
= Control animals
= Animals treated with DEET
= Animals treated with Permethrin
= Animals treated with both DEET and Permethrin
Dentate Gyrus
CA1 Subfield
CA3 Subfield
Cerebellum
B
Density of dying neurons/mm2
1000
750
500
250
0
Dentate Gyrus
CA1 Subfield
CA3 Subfield
Cerebellum


A1
ML
GCL
B1
A2
ML
GCL
B2
A3
ML
GCL
B3
A4
ML
GCL
B4

Appendix 2
DAMD# 17-99-1-9020
Mohamed B. Abou-Donia

STRESS AND COMBINED EXPOSURE TO LOW DAILY DOSES OF PYRIDOSTIGMINE BROMIDE, DEET, AND PERMETHRIN IN ADULT RATS LEADS TO BLOOD BRAIN BARRIER DISRUPTION AND NEUROCHEMICAL AND NEUROPATHOLOGICAL ALTERATIONS IN THE BRAIN.

ABSTRACT

A combined exposure to high doses of pyridostigmine bromide (PB), *N,N*-diethyl *m*-toluamide (DEET), and Permethrin leads to a significant toxicity and neurological dysfunction (Abou-Donia et al., J. Toxicol. Environ. Health, 48: 35-56, 1996). We investigated the effects following combined exposure to low doses of these chemicals with stress, simulating the daily exposure experienced by veterans to these chemicals during Persian Gulf War. Two groups of male Sprague-Dawley rats were administered PB (1.3mg/kg/d, oral), DEET (40mg/kg/d, dermal), and permethrin (0.13mg/kg/d, dermal) for 28 days. Animals in one of the above two groups were subjected to stress every day for the duration of the experiment by placing them in a Plexiglas® restraint tube for 5 minutes. Two additional groups of animals (one subjected to stress and vehicle treatment, and another treated with vehicle alone) served as controls. Three sets of five animals from each of the above four groups were processed for: 1) evaluation of the blood brain barrier (BBB) permeability using injections of [^{3}H]hexamethonium iodide and 10% type IV horseradish peroxidase (HRP); 2) acetylcholinesterase (AChE) activity and m2 muscarinic ACh receptor biochemical assays; and (3) Hematoxylin and Eosin (H&E) staining and microtubule associated protein-2 (MAP-2) immunostaining. Animals subjected to either chemical treatment or stress alone did not show changes in body weight, brain [^{3}H]hexamethonium iodide uptake, brain AChE, plasma ChE but exhibited a slight increase in BBB permeability by HRP and a decreased m2- muscarinic ACh receptor ligand binding, in comparison to control animals. In addition, these animals exhibited either no or minimal neuronal cell death. In contrast, animals subjected to both chemical treatment and stress exhibited a dramatic increase in BBB permeability (with focal perivascular accumulation of HRP in both cerebrum and the brainstem), a significant decrease in brain AChE activity, a decrease in m2 muscarinic ACh receptor ligand binding density in midbrain and cerebellum, and a significant neuronal cell death associated with a reduced MAP-2 expression in the cerebral cortex and the hippocampus. These results underscore that, when combined with stress, exposure to even low doses of PB, DEET, and permethrin, that produce minimal effects by themselves, leads to a significant brain injury.

INTRODUCTION

Since their return from the war, some Persian Gulf War (PGW) veterans have complained of symptoms including chronic fatigue, muscle and joint pain, ataxia, rash, headache, difficulty in concentrating, forgetfulness, and irritability (Institute of Medicine, 1995). During the war, the veterans were exposed to a unique combination of biological, chemical, and psychological environments. Combinations of chemical exposures included a variety of pesticides such as DEET and permethrin (Institute of Medicine, 1995). Additionally, these veterans were given a course of 21, 30mg tablets of pyridostigmine bromide (PB) as prophylactic treatment to protect against organophosphate nerve agents (Persian Gulf Veterans Coordinating Board, 1995). PB is known to be relatively safer at the given dose. PB, a quaternary dimethyl carbamate was given to veterans for prophylactic protection to shield acetylcholinesterase (AChE) from the nerve agent poisoning by reversibly inhibiting 30-40% of the AChE in the peripheral nervous system, thus protecting the enzyme from irreversible inhibition by nerve agents (Blick *et al.,* 1991). The enzyme activity is restored following spontaneous decarbamylation resulting in near normal neuromuscular and autonomic functions (Blick, *et al.,* 1991). Toxic symptoms associated with PB overdose are a result of over stimulation of nicotinic and muscarinic receptors in the peripheral nervous system resulting in exaggerated cholinergic effects such as muscle fasciculations, cramps, weakness, muscle twitching, tremors, respiratory difficulty, gastrointestinal tract disturbances and paralysis (Abou-Donia *et al.,* 1996). Central nervous system effects of PB are not observed unless BBB permeability is compromised because PB does not cross the BBB owing to the positive charge on the quaternary pridinyl nitrogen (Birtley, *et al.,* 1966).

N, N-Diethyl *m*-toluamide (DEET) is used as an insect repellant against mosquitoes, flies, ticks and other insects in the form of lotion, stick or spray (Robbins and Cherniack, 1986, McConnell *et al.,* 1986). Extensive and repeated topical application of DEET leads to human poisoning including death (Gryboski *et al.,* 1961; Roland *et al.,* 1985; Edwards and Johnson, 1987). The symptoms associated with DEET

poisoning are characterized by tremors, restlessness, difficulty in speech, seizures, impairment of cognitive function and coma (McConnell *et al.,* 1986). Extremely high levels of DEET exposure have been reported to cause spongiform myelinopathy (Verschoyle *et al.,* 1992). Because DEET efficiently crosses the dermal barrier (Windheuser *et al.,* 1982; Hussain and Ritchel, 1988) and localizes to dermal fat deposits (Blomquist and Thorsell, 1977: Snodgrass *et al.*, 1982), it is likely that DEET may enhance the availability of drugs and toxicants in other organs and cause regulatory changes such as changes in blood brain permeability. Permethrin is a type I synthetic pyrethroid insecticide that exists in four different sterioisomers (Casida *et al.,* 1983). Its insecticidal activity persists for several weeks following a single application. Permethrin intoxication results as a consequence of modification of sodium channel such that it remains open for a longer time, leading to repetitive discharges after single stimulus (Narahashi, 1985). This repetitive nerve action is associated with tremor, hyperactivity, ataxia, convulsions, and in some cases to paralysis.

We have previously reported that concurrent exposure to large doses of PB with DEET and permethrin results in increased neurotoxicity in hens (Abou-Donia et al, 1996). In the present study, we investigated the neurotoxic effects following combined exposure to low doses of these chemicals with stress, simulating the daily exposure experienced by veterans to these chemicals during Persian Gulf War. Analyses of neurotoxic effects included evaluation of BBB permeability, quantification of plasma BChE, brain AChE, and m2 muscarinic ACh receptor ligand binding in different brain regions, and assessment of neuronal cell death and microtubule associated protein-2 (MAP-2) expression in soma and dendrites of neurons. Our results clearly suggest that, when combined with stress, exposure to even low doses of the above chemicals leads to significant increases in BBB permeability, significant decreases in brain AChE and m2 muscarinic ACh receptor ligand binding, and a diffuse neuronal cell death and cytoskeletal abnormalities in the cerebral cortex and the hippocampus.

MATERIALS AND METHODS

Chemicals

Technical-grad (93.6%) permethrin (±)-Cis/trans-,3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester was obtained from Roussel Uelaf Corporation (Pasadena, TX). DEET (≥97% *N,N*-diethyl *m*-toluamide), pyridostigmine bromide (≥ 99%, 3-dimethylamino carbonyloxy *N*-methylpridinium bromide), acetylthiocholine iodide, butyrylthiocholine iodide, horseradish peroxidase type IV and diaminobenzidine tetra hydrochloride was purchased from Sigma (St. Louis, MO). [*N*-methyl-^{3}H]hexamethonium iodide (sp. activity 18Ci/mmol) were obtained from US Army Medical Research Institute for Chemical Defense (USAMRICD, MD). [^{3}H]AF-DX384 (sp. activity 106.5 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All other chemicals and reagents were of highest purity available from commercial sources.

Animals

Male Sprague-Dawley rats weighing 225-250g were obtained from Zivic-Miller Laboratories, Allison Park, PA. The animals were randomly assigned to control and treatment groups and housed at 21-23°C with a 12-h light/dark cycle. They were supplied with Purina Certified Rodent Chow (Ralston Purina Co., St. Louis, MO), and tap water ad libitum. The rats were allowed to adjust to their environment for a week before treatment. Animal care was in accordance with institutional guidelines and approved by Duke University Animal Care and Use Committee.

Treatment Protocol

Animals in Group 1 (n = 15) were treated with PB (1.3mg/kg/d, oral in water), DEET (40 mg/kg/d, dermal in 70% ethanol) and permethrin (0.13mg/kg/d, dermal in 70% ethanol) for 28 days. Animals in Group 2 (n = 15) were treated with all of the above chemicals and also subjected to 5 minutes of restraint stress everyday for the duration of experiment following the chemical treatment by placing them in a Plexiglas® cylinder. Animals in Group 3 (n = 15) were treated with dermal application of 70%

ethanol and oral water (1ml/kg) and subjected to stress as described in Group 2. Animals in Group (n=5) 4 were treated with a dermal application of 70% ethanol and oral water daily for 28 days.

Analysis of Blood brain barrier permeability using [^{3}H]hexamethonium iodide uptake assay

Twenty-four hours after the last treatment, subgroups of five animals in each group were anesthetized with sodium Nembutal (100 mg/kg) and intravenously (i.v.) injected with [^{3}H]hexamethonium iodide (10 μCi, mixed 1:1 with cold hexamethonium iodide to give a final dose of 0.71 mg/kg, 1μ Ci/kg). After 10 minutes, the blood was collected from the heart with heparinized syringes and the animals were sacrificed by decapitation. Brains were removed and placed in ice-cold normal saline. Brain regions (cortex, brainstem, midbrain and cerebellum) were rapidly dissected on ice and frozen immediately using liquid nitrogen. The plasma was separated from whole blood by centrifugation. Both plasma and brain regions were stored at -20°C until further analyses. Regions of cerebral cortex, brainstem, midbrain and cerebellum (50 – 100 mg) and 100 μl of plasma were oxidized in a tri-carb Packard Model 306B tissue oxidizer (Packard Instrument Co., Downers Grove, IL), using the trapping solution Monophase and the scintillation fluid Permaflour V followed by counting in a liquid scintillation spectrometer (Beckman Instruments Corp., Palo Alto, CA) for 5 minutes. Radioactivity counts were corrected for dilution, quenching, background, and counting efficiency. Counts were recorded as dpm/g of tissue or 1 ml plasma and compared with control level. Data are presented as ratio of Tissue/Plasma.

Analysis of Blood brain barrier permeability using horseradish peroxidase

Five animals in each group were injected HRP type VI (10 % solution in saline) at a dose of 135 mg/kg (i.v.) to give a final concentration of 2 mg/animal. Thirty minutes following injection of HRP, animals were perfused with a fixative containing 4% paraformaldehyde and 2% gluteraldehyde in phosphate buffered saline (PBS). Brains were removed, post-fixed, embedded in paraffin, and sections cut (8 micron thick) using a microtome. Serial sections through the brain were deparaffinized, and processed for HRP visualization by incubating in diaminobenzidine (0.05%) and hydrogen peroxide (0.01%) solution in Tris-Water for 15 minutes. Following extensive washing in PBS, and sections were osmicated

using 1% osmium tetroxide for 30 minutes, dehydrated, and mounted. Breached and non-breached areas of HRP stained sections were analyzed by light microscopy using dark field optics to clearly visualize the localization of HRP. In addition to the above analysis, representative sections were also processed for HRP immunohistochemistry. For this, sections were deparaffinized, blocked with 10% goat serum in 0.1M PBS for 20 minutes and incubated with primary antibody (Sigma) for 45 minutes at 40^0C. Sections were then incubated with appropriate secondary antibody solution in 0.1M PBS for 20 minutes. Sections were developed by the ABC method of Hsu et al., (1981) using reagents from Vector Labs (Burlingame, CA, USA).

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) Assays

Brain (AChE) and plasma (BChE) activities were determined according to the method of Ellman et al., (1961) but modified for assay in a Molecular Devices VERSAMax Tunable Microplate Reader (Molecular Dives Corp., Sunnyvale, CA) as previously described (Abou-Donia et al, 1996). Protein concentration was determined by the method of Smith et al., (1985).

Muscarinic Acetylcholine Receptor (mAChR) Binding Assay

For the assay of m2 muscarinic AChR, the tissue was homogenized in 10 mM phosphate buffer, pH 7.4 and centrifuged at 40,000 x g for 10 minutes and the membranes were suspended in the same buffer at the protein concentration of 1.5-2.5 mg/ml, as described by Huff et al., (1994). The m2 muscarinic AChR binding was carried out by using m2-selective ligand, [^{3}H]AFDX 384 at room temperature for 60 minutes, as described by Slotkin et al (1999).

Histopathological analyses

Following exposure regimen (i.e., on day 29), five animals from each group were anesthetized with pentobarbital (100 mg/kg) and perfused through the heart with saline followed by 4% paraformaldehyde and 0.1% gluteraldehyde in Tris buffer. The brains and livers were removed, post-fixed, and embedded in paraffin according to standard histological techniques. Five to six micrometer thick coronal sections were cut through different brain regions. In every brain (n=5), representative

sections (n = 5) through the motor and somatosensory cortex, the septal hippocampus, and liver were processed and stained with hematoxylin and eosin (H & E) for light microscopic observation.

Microtubule associated protein-2 (MAP-2), Glial fibrillary acidic protein (GFAP), and Lectin immunohistochemistry

Additional sections from brain were immunohistochemically stained using antibodies for MAP-2 (1:1000 dilution; Sternberger Monoclonals), glial fibrillary acidic protein (GFAP, 1:10,000; Dako), and Lectin (1:500; Vector) with the ABC method (Hsu et al., 1981), using reagents from Vector Labs (Burlingame, CA).

Quantitative evaluation of the number of surviving and dying neurons in different brain regions:

Numerical density of surviving and dying neurons per mm^2 area of tissue in H & E stained sections was measured for layers III and V of the motor cortex, granule cell layer of the dentate gyrus, and pyramidal cell layer of CA1 and CA3 subfields of the hippocampus. Five sections through each of the above brain regions were employed for these measurements in each animal belonging to the following four groups: (a) control animals (n = 5); (b) animals treated with stress alone (n = 5); (c) animals treated with chemicals alone (n = 5); and (d) animals treated with both stress and chemicals (n = 5). Measurements in sections from various groups were performed in a blinded fashion using experimental codes. The coding was such that animal treatments were not known during measuring; however, sections that came from the same animal were identified. All measurements were performed using a Nikon E600 microscope equipped with eyepiece grid. At a magnification of 400X (using 40X objective lens and 10X eyepieces), both surviving and dying neurons within a unit area of each section (measuring 18,750 μm^2 for layer III of cortex, 62,500 μm^2 for layer V of the cortex, 12,500 μm^2 for dentate granule cell layer, 6,250 μm^2 for CA1 pyramidal cell layer, and 12,500 μm^2 for CA3 pyramidal cell layer) were counted. For measurement of surviving neurons, only those which exhibited hematoxylin

stained nucleus with a clear nucleolus were counted. For measurement of dying neurons, only those neurons that exhibited dense eosinophilic staining in both soma and proximal dendrites were counted. Finally, the density of neurons per unit area was transformed to the numerical density per mm^2 area of respective brain region.

Statistical Analyses

The mean value for each of the five brain regions (layers III and V of the motor cortex, granule cell layer of the dentate gyrus, and CA1 and CA3 pyramidal cell layers of the hippocampus) was calculated separately for each animal by using data from 5 sections before the means and standard errors were determined for the total number of animals (n=5) included per group. Mean values between different groups of animals were compared separately for each of the above brain regions using one-way ANOVA with Student's Newman-Keuls multiple comparison post-hoc test.

RESULTS

General observations

The clinical condition of animals did not show any difference among different treatment groups or between treatments groups and control. Figure 1 presents body weight data in adults rat treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal) and permethrin (0.13 mg/kg, dermal) with or without stress and stress alone for 28 days. Rats treated with both chemicals and stress showed weight loss compared to controls between weeks 2 to 4 (8 to 14% decrease, $p < 0.01$) and compared to animals treated with stress alone between weeks 1 to 2 ($p < 0.05$).

[Figure 1 here]

Effects on BBB permeability

We evaluated the effect of combined exposure to daily doses of PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal) and permethrin (0.13 mg/kg, dermal) for 28 days with and without stress on BBB permeability. Exposure to either chemicals or stress, alone had no significant effect on BBB permeability (Fig. 2). In contrast, combined exposure to chemicals and stress led to a significant increase in BBB permeability in cerebral cortex, cerebellum ($p < 0.01$; Fig. 2) and the midbrain ($p < 0.05$; Fig. 2). The increase in BBB permeability in cerebellum was ~366% of control animals, suggesting that cerebellum is the most susceptible brain area for BBB disruption following exposure to the combination of chemicals and stress, as assessed by [^{3}H]hexamethonium iodide uptake.

BBB permeability was also assessed by the penetration of horseradish peroxidase HRP) into the parenchyma of the CNS as vesicles positive for HRP using DAB staining (Fig. 3). Exposure to chemicals or stress alone resulted in minimal perivascular penetration of HRP (Fig. 3 [A1, A2]). Whereas, co-exposure with chemicals and stress resulted in abundant HRP positive vesicles in cerebral cortex, cerebral white matter such as corpus callosum, deep gray nuclei and brain stem (Fig. 3 [B2]) compared to animals

treated with chemicals alone (Fig. 3 [B1]). In this group, five out of five animals (100%) exhibited significant penetration of HRP into the brain parenchyma. Further evaluation of the distribution of HRP in brain using HRP immunohistochemistry revealed a large number of HRP positive neurons in animals treated with both stress and chemicals, suggesting movement of HRP molecules to brain regions by vesicular transport through the endothelium. A dense vesicular staining of HRP in the cytoplasm, abluminal surface, basement membrane, extra-cellular spaces of adjacent neuropil, and endothelial tight junctions in animals treated with both chemicals and stress (figure not shown).

Since increased BBB permeability results in microglial activation and hypertrophy, we investigated the hypertrophy of microglial cells by immunohistochemical detection of lectin. A very extensive lectin binding around capillaries and prominent patches of diffuse lectin staining was evident in the brain parenchyma of animals exposed to both chemicals and stress (Fig. 4 [B]). Furthermore, processes of microglial cells in animals exposed to combination of stress and chemicals exhibited increased complexity, suggesting that microglial cells undergo hypertrophy following exposure to both chemicals and stress.

[Figures 2, 3, and 4 here]

Effects on AChE activity in brain regions and BChE acdivity in plasma

AChE in cerebral cortex, brainstem, midbrain, and cerebellum and BChE activities in plasma were assayed and the results are presented in Fig. 5. Only combined exposure to chemicals and stress caused a significant decrease in AChE activity in midbrain, brainstem, and cerebellum compared to control animals (65%, 71%, and 64% of controls; $p < 0.05$). Plasma BChE activity was also significantly inhibited (66% of controls) by combined exposure to chemicals and stress ($p < 0.05$). In addition, animals treated with chemicals and stress exhibited a significant decrease compared to animals treated with stress alone in midbrain, cerebellum, and plasma ($p < 0.05$).

[Figure 5 here]

Effects on m2-muscarinic ACh receptors

Muscarinic ACh receptor down–regulation was very evident in animals treated with both chemicals and stress. We carried out ligand binding studies for m2-muscarinic ACh receptor in cerebral cortex, midbrain, brainstem, and cerebellum using m2-selective ligand, [^{3}H]AF-DX-384 (Fig. 6). Animals treated with chemicals and stress exhibited a significant decrease compared to controls in cortex and cerebellum ($p < 0.05$); Fig. 6. Animals treated with chemicals and stress also exhibited a significant decrease compared to animals treated with stress alone in midbrain, and cerebellum ($p < 0.05$). Animal treated with chemicals alone exhibited a significant decrease compared animals treated with stress alone in midbrain ($p< 0.05$). Animals treated with stress alone exhibited a significant decrease compared to controls in cortex and brainstem ($p < 0.05$; Fig. 6).

[Figure 6 here]

Histopathological changes in the liver

Examination of sections through the liver stained with H & E showed that a significant alteration in hepatic cytoarchitecture, characterized by portal and peri-portal fibrosis with mononuclear inflammatory cells occurs following exposure to both stress and chemicals (Fig. 7). In addition, sinusoidal dilatation, markedly diffuse steatosis, predominant microvacuoles were observed in animals treated with both chemicals and stress, in comparison to control animals (Fig. 7). The above alterations in hepatic cytoarchitecture following exposure to both stress and chemicals were highly consistent (100%) between animals. In contrast, in animals treated with chemicals alone, only one out of five animals (20%) exhibited alterations. Stress caused no effect on the liver.

[Figure 7 here]

Histopathological changes in the brain

Evaluation of brain sections stained with H & E clearly revealed neuronal degeneration in rats treated with both chemicals and stress, in comparison to animals treated with chemicals, stress, or vehicle alone. Degenerating neurons were characterized by eosinophilic staining of both cell body and proximal dendrites. In contrast, the healthy neurons in the same section exhibited hematoxylin stained nucleus (with a clear nucleolus) within a lightly eosin stained perinuclear cytoplasm. The brain regions where neuronal degeneration was most obvious include motor and somatosensory areas of the cerebral cortex, and dentate gyrus, CA1 and CA3 subfields of the hippocampus. Other areas of the brain, though showed occasional dying (eosinophilic) neurons, the overall cytoarchitecture remained comparable to animals treated with chemicals, stress, or vehicle alone.

Alterations in the cerebral cortex

In animals treated with chemicals and stress, both superficial and deeper regions of motor and somatosensory cortex exhibited degenerating neurons in H & E stained sections. In superficial region (layers I-III; Fig. 8), degenerating neurons were conspicuous in layer III. Majority of degenerating neurons in this layer were of pyramidal type with prominent eosinophilic apical dendrites (Fig. 8 [A4]. In deeper regions of the cortex (layers IV-VI), degenerating neurons were mostly observed in layer V. These are larger pyramidal neurons with prominent apical and basal dendrites emanating from a larger pyramidal-shaped cell body (Fig. 9 [A4]).

The MAP-2 immunostaining in animals treated with either vehicle or stress, alone showed normal distribution of MAP-2 within dendrites and neuronal perikarya of both superficial and deeper regions of the cerebral cortex (Figs. 8 [B1, B2] and 9 [B1, B2]). Treatment with chemicals alone caused a slight reduction of MAP -2 immunostaining (Figs. 8 [B3] and 9 [B3]). In contrast, in animals treated with chemicals and stress, the overall MAP-2 immunostaining representing dendrites and soma of neurons was dramatically reduced (Figs. 8 [B4] and 9 [B4]). However, in layer V of the cerebral cortex, neurons

having positive MAP-2 immunostaining exhibited greatly enhanced expression of MAP-2 in both soma and dendrites (Fig. 9 [B4]).

[Figures 8, and 9 here]

Quantification of surviving and dying neurons per mm^2 area of layers III and V of the motor cortex revealed the following. (1) Animals treated with a combination of stress and chemicals exhibited a significant decrease in the number of surviving neurons in both layers III and V ($p < 0.01$; Fig. 10), in comparison to control animals, and animals treated with stress or chemicals alone. In contrast, animals treated with chemicals alone exhibited a decrease in the number surviving neurons in only the layer V ($p < 0.05$; Fig. 10). The degree of reduction was also less pronounced in this group, compared to animals treated with both stress and chemicals. (2) Animals treated with a combination of stress and chemicals exhibited a significant increase in the number of dying neurons within both layers III and V ($p < 0.001$; Fig. 10) of the motor cortex, in comparison to all other animal groups (control animals, animals treated with stress, alone, and animals treated with chemicals alone). Thus, quantitative analyses of both surviving and dying neurons in the cortex clearly demonstrate a significant loss of neurons with exposure to a combination of stress and chemicals, a moderate loss of neurons with exposure to chemicals alone, and clearly no loss of neurons with exposure to stress alone.

[Figure 10 here]

Alterations in the hippocampal formation

Neuronal degeneration was obvious in the dentate gyrus, CA1 and CA3 subfields of the hippocampal formation following combined exposure to chemicals and stress (Figs. 11, 12 and 13). In dentate gyrus, degenerating neurons were observed in the granule cell layer (Fig. 11[A4]). In CA1 and

CA3 subfields of the hippocampus, degenerating neurons were conspicuous in the stratum pyramidale (Figs. 12[A4] and 13[A4]).

Neuronal cell death in dentate granule cell layer correlated with enhanced GFAP immunoreactivity in both molecular layer and hilus of the dentate gyrus (Fig. 11[B4]). Whereas, neuronal cell death in CA1 and CA3 subfields correlated with a clearly enhanced GFAP immunoreactive reactive astrocytes in strata oriens, and radiatum of CA1 and CA3, likely due to degeneration of dendrites in these strata (Figs. 12 [B4] and 13 [B4]). Control animals and animals treated with stress alone exhibited neither neuronal cell death nor enhanced GFAP immunoreactivity in the hippocampus (Figs. 11, 12, and 13 [A1, A2, B1, B2]). However, animals treated with chemicals alone demonstrated occasional neuronal cell death particularly in the CA3 subfield (Fig. 13[A3]). In addition, these animals exhibited increased GFAP immunoreactivity in all regions of the hippocampus in comparison to vehicle treated control animals and animals treated with stress alone (Figs. 11, 12, 13 [B1, B2, B3]), suggesting that some hippocampal injury occurs following exposure to chemicals alone. However, unlike animals treated with both chemicals and stress, this neural injury is not characterized by significant neuronal cell death.

Evaluation of MAP-2 immunostaining revealed significantly reduced MAP-2 positive dendrites in animals treated with both chemicals and stress but not in animals treated with either chemicals or stress alone. Alterations in MAP-2 expression were particularly conspicuous in the CA1 stratum radiatum. Thus, combined exposure to both chemicals and stress induces a significant neuronal cell death, astrocytic hypertrophy, and cytoskeletal abnormalities (characterized by reduced overall MAP-2 expression) in the hippocampus.

Quantification of surviving and dying neurons per mm^2 area of granule cell layer of the dentate gyrus and pyramidal cell layer of subfields CA1 and CA3 demonstrated the following. (1) Animals treated with a combination of stress and chemicals exhibited a significant decrease in the number of surviving neurons, in comparison to control animals (dentate gyrus, $p < 0.001$; CA1 subfield, $p < 0.05$;

and CA3 subfield, $p < 0.01$; Fig. 14), and animals treated with either stress or chemicals (dentate gyrus, $p < 0.001$; CA1 subfield, $p < 0.05$; and CA3 subfield, $p < 0.01$; Fig. 14). (2) Animals treated with a combination of stress and chemicals exhibited a significantly greater number of dying neurons compared to control animals (dentate gyrus, $p < 0.001$; CA1 and CA3 subfields, $p < 0.05$; Fig. 14), and animals treated with either stress or chemicals ($p < 0.05$ for all regions; Fig. 14). Thus, quantitative analyses of both surviving and dying neurons in the hippocampal formation clearly demonstrate a significant loss of neurons with exposure to a combination of stress and chemicals, and minimal loss of neurons with exposure to either stress or chemicals.

[Figures 11, 12, 13, and 14 here]

DISCUSSION

The symptoms pertaining to abnormalities in central nervous system (CNS) function described by Persian Gulf War (PGW) veterans may have resulted from repeated exposure to a combination of chemicals such as pesticides and prophylactic medications (Institute of Medicine, 1995). Additionally, it has also been suggested that stress may have played a role in the development of symptoms described by PGW veterans. The present study was carried out in rats to evaluate the neurotoxicity following combined exposure to doses low enough to be representative of human exposure levels of PB, DEET and permethrin with stress for 28 days, simulating the daily exposure experienced by veterans to these chemicals during Persian Gulf War.

The principal finding of this study is that a combined exposure to stress and low doses of chemicals PB, DEET, and permethrin in adult rats leads to a widespread neurochemical and neuropathological alterations in the brain. Animals subjected to both chemical treatment and stress exhibited a reduced body weight, a dramatic increase in BBB permeability with focal perivascular accumulation of HRP in cerebrum and the brainstem, a significant decrease in brain AChE activity, and a decrease in m2 muscarinic ACh receptor ligand binding density in midbrain and cerebellum. Further, a diffuse neuronal cell death with a reduced expression of MAP-2 in surviving neurons was observed in the cerebral cortex and the hippocampus. In contrast, animals subjected to either chemical treatment or stress alone did not show changes in BBB [^{3}H]hexamethonium iodide uptake, brain AChE, plasma ChE but exhibited a slight increase in BBB permeability by HRP and a decreased m2- muscarinic ACh receptor ligand binding in cerebellum, cerebral cortex and the brainstem, in comparison to control animals. In addition, these animals exhibited either no or minimal neuronal cell death. However, animals treated with chemicals alone exhibited increased GFAP expression and a slightly decreased MAP-2 immunoreactivity, suggesting a mild neural injury with exposure to low doses of chemicals alone. Collectively, the above results underscore that, when combined with stress, exposure to even low doses of PB, DEET, and permethrin leads to a significant brain injury, characterized by dramatically enhanced BBB permeability,

altered acetylcholine function, diffused neuronal cell death, and cytoskeletal abnormalities in surviving neurons.

Extent of alterations following exposure to low doses of PB, DEET and permethrin, and stress.

Animals that received both chemicals and stress exhibited a significant decrease in body weight (14%) and widespread alterations throughout the CNS. Firstly, the permeability of BBB exhibited a significant increase in many areas of the brain particularly cerebral cortex, midbrain, cerebellum and corpus callosum. The disruption of BBB in these regions was characterized by excessive accumulation of HRP outside capillaries. Secondly, these animals exhibited a significant decrease in plasma BChE activity and brain AChE activity in midbrain, brainstem, and cerebellum. Thirdly, there was a significant decrease in m2 muscarinic AChR ligand binding in midbrain of these animals. Furthermore, the above alterations in animals treated with chemicals and stress were associated with neuronal cell death and a significantly enhanced astrocytic GFAP expression (suggestive of glial hypertrophy) in many regions of the brain. The regions where these neuropathological alterations were conspicuous include the motor and somatosensory regions of the cerebral cortex, and the dentate gyrus and CA1 and CA3 subfields of the hippocampus. In cerebral cortex, the neuronal cell death was more pronounced in pyramidal neurons belonging to layers III and V of the motor cortex, pyramidal and granule neurons belonging to layers III and V of the somatosensory cortex, granule cells of the dentate gyrus, pyramidal neurons of hippocampal CA1 and CA3 subfields. In addition, these regions also demonstrated abnormalities in the expression of cytoskeletal proteins such as MAP-2. The dendrites of surviving neurons clearly had reduced MAP-2 protein. The abnormalities in MAP-2 expression in some of the surviving neurons (particularly apical dendrites of cortical pyramidal neurons in layer III) were characterized by a beaded, disrupted, or wavy appearance. The GFAP expression in this group was exemplified by GFAP expression in soma of astrocytes and

hypertrophy of astrocytic processes emanating from the soma. Thus, there is clearly a more pronounced neural injury following exposure to both chemicals and stress in combination than exposure to either chemicals or stress alone. This differential neural damage between these groups likely reflects differences in the permeability of BBB following various treatments. As exposure to both chemicals and stress significantly increases the permeability of BBB, the capability of these rats for detoxification and elimination of chemicals is likely to be compromised significantly resulting in a much greater concentration of chemicals crossing the BBB and reaching different target sites in the brain. A significant inhibition of both brain AChE and plasma BChE activity in association with liver injury strongly supports the above contention. The liver injury was manifested as sinusoidal dilation, markedly diffuse steatosis, predominantly micro vacuolar, mild portal, and periportal fibrosis and infiltration of mononuclear inflammatory cells.

Previous studies have shown that exposure to higher doses of PB, DEET, and permethrin leads to increased indices of toxicity and more severe brain damage, characterized by neurological dysfunction and neuropathological changes (Abou - Donia et al., 1996; 2001a). Recently, we have demonstrated that the test-compounds at the same regiments used in the present study, caused impairment of locomotor and sensorimotor performance in treated rats (Abou-Donia et.al. 2001b) In these latter studies, the most severe brain damage was found in the cerebral cortex, and the hippocampus. The focus of the present study was to evaluate effect of lower doses of chemicals in combination with stress using various markers of neuropathology. Our results clearly indicate that even at low doses, exposure to chemicals in combination with stress induces neuronal cell death. Although the overall neuronal cell death is less than following exposure to higher concentration of chemicals and stress, this low dose exposure to chemicals and stress does induce significant cytoskeletal abnormalities in surviving neurons, as revealed by dramatically reduced MAP-2 expression and glial hypertrophy in many brain regions. The glial hypertrophy was characterized by the presence of reactive astrocytes and increased microglial activity.

Both astrocytes and microglia are involved in brain function under both normal and pathological conditions (Dickson et al, 1993, McGeer et al, 1993). Following brain injury astrocytes exhibit hypertrophy and become reactive astrocytes, and microglial cells transform from resting state to activated state, which is characterized by amoeboid shape. Activated microglial cells also proliferate and engulf degenerating elements such as debris following neuronal degeneration (Giulian et al, 1989, Stoll et al, 1989).

Potential reasons for alterations in BBB following exposure to both chemicals and stress.

Increased BBB permeability following to chemicals and stress is likely to result from a direct effect of chemicals and stress on cerebrovascular endothelial cells. These cells form and maintain the lining of the barrier that protects the CNS from unwanted or noxious agents (Joo, 1996). Several regulatory molecules such as *P*-glycoprotein, adheren and other neural adhesion molecules are abundantly expressed on the endothelial lining of cerebral vasculature (Joo, 1996). These molecules maintain tight gap junction, allowing regulated flow of ions, molecules required for maintaining the neuronal homeostasis. Co-exposure to chemicals and stress may induce changes in the arrangement and topography of gap junction molecules, thereby allowing opening of the junction and resulting in an increased permeability of the BBB. Alternatively, combined exposure may inhibit the *P*-gp pump function allowing enhanced access of otherwise non-permeable molecules. The precise biochemical events leading to the increased permeability in BBB following exposure to chemicals and stress remains to be elucidated. Several earlier studies, however, have suggested that a variety of acute stress such as social stress, restraint stress, forced swimming, and heat increase can enhance BBB permeability in both rats and mice (Sharma et al., 1991, Sharma and Dey, 1986, and 1987, Belova and Jonsson, 1982, Skultetyova et al., 1998, Wijsman and Shivers et al., 1993). It has been suggested that involvement of various mediators play a role in changes of BBB permeability following stress. Melia et al., (1994) and Friedman et al., (1996) have shown that immediate early genes such as induction of c-Fos might play a role in stress- resulting from increased BBB permeability. Further, chronic restraint stress induces decreases in aminergic and cholinergic induced increase in BBB permeability and neurotoxicity

neurotransmission (Sunanda et al., 2000). Stress can also lead to increases in the activation of glutamatergic neurotransmission resulting in excessive release of glutamate in the hippocampus, prefrontal cortex and basal ganglia (Moghaddam et al., 1994; Stein – Behrens et al., 1992; Watanabe et al., 1992; Moghaddam et al., 1993). A greatly enhanced level of glutamate is detrimental as it can cause neurodegeneration (Armanini et al., 1990; Headley et al., 1990).

Relationship between neural injury and cytoskeletal changes

The cytoskeletal abnormalities in both cerebral cortex and the hippocampus suggest injury to these neurons. The type of neuronal damage in terms of MAP-2 expression is consistent with previous studies following exposure to various neurotoxic chemicals (Kitagawa et al., 1989; Petrall et al., 1991; Chauhan et al., 1991 and 1992; Neelima et al., 1993). Previous studies also indicate that the reduction of MAP-2 expression in neurons following excitotoxic brain damage is likely related to alterations in the concentration of intercellular calcium. This is because increased intracellular calcium concentration can mediate alterations of MAP-2 expression both by influencing the activity of kinases, phosphatases, all of which can rapidly proteolyze MAP-2 and dramatically reduce the amount of MAP-2 within neurons. Also, an increased intracellular calcium concentration can lead to an excessive degradation of MAP-2 by calpain and result in neuronal dysfunction (Johnson and Jope, 1992; Saatman et al., 2001). Since the most consistent abnormalities involving in the cerebral cortex of individuals with mental retardation (MR) is a reduction in dendritic arborizations (Kaufmann, 1996; Kaufmann and Moser, 2000), reduced MAP-2 expression can lead to regression of dendritic arborization and compromise the function of the involved area. Further, an increased accumulation of MAP-2 in soma and proximal dendrites and a decreased expression of MAP-2 in distal dendrites suggest alterations in transport of microtubules. This change can lead to destabilization of dendrites and result in aberrant functioning of neurons particularly loss of synapses due to resorption of post-synaptic specializations such as dendritic spines. It remains to be seen whether these injured neurons recover over time or undergo progressive degeneration. Both degeneration of neurons and reductions in the expression of MAP-2 in surviving neurons of layers III and V of the motor cerebral cortex and the hippocampal formation are of significant importance. This is because

layers III and V neurons in the cortex are the source of axons of the corticospinal tract, which is the largest descending fiber tract (or motor pathway) from the brain controlling movements of different contralateral muscle groups. Thus, significant death of layer V neurons of the motor cortex following combined exposure to chemicals and stress could lead to weakness and loss of strength. Further, disruption of hippocampal circuitry due to degeneration of neurons in different subfields can lead to learning and memory deficits. The above changes explain some of the symptoms such as loss of memory, muscle weakness, and alterations in learning ability observed in Gulf War Veterans.

Potential reasons for changes in AChE activity and m2 muscarinic ACh receptor binding following exposure to both chemicals and stress

Among different chemicals used in this study, PB is known to inhibit the peripheral AChE, by this shielding it from nerve agents. The possible mechanisms of DEET-induced neurotoxicity are unknown, but it has been shown to cause demyelination leading to spongiform myelinopathy of cerebral nuclei (Verschoyle *et al.,* 1992) at extremely high dose. Permethrin-induced PNS defects involve the modulation of axonal and muscle sodium channel so that the channel remains open for a longer period, resulting in the hyperexcitability of the nervous system and muscles (Narahashi, 1985). As exposure to combination of chemicals alone did not cause any significant change in cholinesterase activity, it is likely that AChE is not a preferred target for these chemicals. It is possible that PB enters the CNS parenchyma because of stress-induced increase in the permeability of the BBB, and the consequent inhibition of the CNS AChE activity likely enhances neurotoxic effects of chemicals. In fact, the recent use of PB in Alzheimer's Disease and conventional use of this chemical for myasthenia gravis (Breyer-Pfaff, et al, 1990) is based on the premise of providing acetylcholine to the neuronal sites already depleted of the acetylcholine and exhibiting defective functioning of nicotinic acetylcholine receptors and neuronal muscarinic receptors. However, in the case of the CNS, exposure to PB in the presence of other neurotoxic agents may have additive effect because of the ability of PB to inhibit CNS AChE as well the regulation of receptor(s) activated by acetylcholine.

Muscarinic acetylcholine receptors in the CNS are comprised of five distinct classes of receptors (m1-m5). These receptors have distinct structural and pharmacological features and show differential cellular localization (Levey et al., 1991). These classes of receptors are coupled to different G-proteins in order to transduce cellular signals from the cell surface. M2-muscarinic ACh receptor is coupled to inhibitory G protein, Gi, leading to the inhibition of adenylate cyclase. Others and we have previously reported that m2-muscarinic receptor is regulated by a variety of organophosphate compounds *in vivo* and *in vitro* (Huff et al., 1994, Ward, et al., 1992). Data in the current study suggest that co-exposure to PB, DEET and permethrin with stress can mediate inhibitory responses by decreasing the m2-muscarinic ACh receptor binding densities in cortex, and midbrain, *in vivo.* These regulatory changes in m2-muscarnic ACh receptor are manifested as a decrease in certain neurobehavioral responses (Abou-Donia et al, 2001b), some of which may be controlled by brainstem and/or midbrain.

Conclusions

The results of this study clearly demonstrate that exposure to stress added to combined exposure to PB, DEET and permethrin at real life doses leads to diffuse neuronal cell death in the cerebral cortex and the hippocampus. In addition, the above exposure paradigm leads to abnormalities in cellular and biochemical parameters of neuronal function in many regions of the CNS. Taken together, these alterations can lead to CNS deficits and neurological symptoms reported by PGW veterans.

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Figure Legends

Figure 1 - Effect of daily administration of pyridostigmine bromide (PB) (1.3mg/kg/d, po), DEET (40mg/kg/d, dermal) and permethrin (0.13mg/kg/d, dermal), with or without stress on body weight of rats. Data are presented as percentages of the initial body weight. Animals were assessed for body weight each week. The percentage of initial body weight for controls (Mean ± SEM) is 134.93 ± 1.895 (Week 1), 149.43 ± 1.428 (Week 2), 160.17 ± 1.157 (Week 3), and 174.09 ± 1.215 (Week 4). Analysis with one-way ANOVA revealed significant differences between groups ($p < 0.001$). The post-hoc analysis with Student's Newman-Keuls multiple comparisons test further showed that animals treated with both chemicals and stress exhibited a significant decrease in body weight, compared to both control animals ($p < 0.01$) and animals treated with stress alone ($p < 0.05$).

Figure 2 - [^{3}H]hexamethonium iodide uptake in rat brain regions (cortex, midbrain, brainstem, and cerebellum), and plasma following daily treatment of animals with pyridostigmine bromide (PB) (1.3mg/kg/d, po), DEET (40mg/kg/d, dermal) and permethrin (0.13mg/kg/d, dermal), with or without stress. Data (Mean ± SEM) are presented as percentage of control animals of the ratio of tissue uptake (DPM/gm) versus that of the plasma (DPM/mL; T/P). Control values are as follows: cortex, 0.100 ± 0.007; midbrain, 0.110± 0.01; brainstem 0.110± 0.015; and cerebellum 0.121 ± 0.014. Analysis with one-way ANOVA revealed significant differences between groups in cerebral cortex ($p < 0.01$), midbrain ($p < 0.05$), and cerebellum ($p < 0.01$). The post-hoc analysis with Student's Newman-Keuls multiple comparisons test further revealed that animals treated with chemicals and stress exhibited a significant increase compared to controls (ANOVA), in all of the above three regions ($p < 0.05$), and animals treated with either stress or chemicals alone ($p < 0.05$).

Figure 3 – Penetration of HRP through the barrier following daily exposure of animals to pyridostigmine bromide (PB) (1.3mg/kg/d, po), DEET (40mg/kg/d, dermal) and permethrin (0.13mg/kg/d, dermal), with

or without stress. A1 and A2 are examples from control rat group (A1) and the group subjected to stress alone (A2). B1 is an example from rats treated with chemicals alone whereas B2 is an example from rats treated with both stress and chemicals. A significant and diffuse breach of the barrier to HRP is clearly evident in the cerebral cortex of the rat treated with both stress and chemicals (B2). Comparison between groups reveals that HRP movement through the endothelium into the brain parenchyma by vesicular transport is maximal following treatment with both stress and chemicals (B2), quite significant following treatment with chemicals alone (B1), moderate followed treatment with stress alone (A2), and absent following treatment with vehicle (i.e. controls, A1). Scale bar, 100 μm.

Figure 4 – Effect of combined exposure on microglial activation in the cerebral cortex. A is an example of lectin immunostaining from a control rat. B is an example of lectin immunostaining from a rat treated daily with pyridostigmine bromide (PB) (1.3mg/kg/d, po), DEET (40mg/kg/d, dermal) and permethrin (0.13mg/kg/d, dermal) with stress. Note extensive lectin binding around capillaries in the latter group (B) (arraw).

Figure 5 - Effect of daily administration of pyridostigmine bromide (PB) (1.3mg/kg/d, po), DEET (40mg/kg/d, dermal) and permethrin (0.13mg/kg/d, dermal) with or without stress on AChE and plasma BuChE activity in different regions of the rat brain (cortex, midbrain, brainstem, and cerebellum). Data (Mean ± SEM) are presented as percentages of corresponding control values (μmol substrate hydrolyzed/min/gm of tissue or ml of plasma). Control values are as follows: cortex, 2.758 ± 0.321; midbrain, 5.510 ± 0.333; brainstem, 3.023 ± 0.247; cerebellum, 1.033 ± 0.0477; and plasma 0.0488 ± 0.004. Analysis with one-way ANOVA revealed significant differences between groups in midbrain, brain stem, cerebellum and plasma ($p < 0.05$). The post-hoc analysis with Student's Newman-Keuls multiple comparisons test further revealed that animals treated with chemicals and stress exhibited a significant decrease compared to controls in all of the above three brain regions and plasma ($p < 0.05$). In brain stem, animals treated with chemicals and stress also exhibited a significant decrease compared to

animals treated with stress alone ($p < 0.05$). Further, BuChE activity in plasma showed a significant decrease in animals treated with chemicals and stress compared to animals treated with stress alone ($p < 0.05$).

Figure 6- Effect of daily administration of pyridostigmine bromide (PB) (1.3mg/kg/d, po), DEET (40mg/kg/d, dermal) and permethrin (0.13mg/kg/d, dermal) with or without stress on brain M2-muscarinc receptor ligand binding in different regions of the brain (cortex, midbrain, brainstem, and cerebellum). Membrane preparation and M2-mAChr - specific ligand binding with [^{3}H] AF-DX - 384 was determined as described in Materials and Methods. Data (Mean ± SEM) are presented as % of corresponding controls. Control values are: cortex 5804.3 ± 318.45 Pmol/mg of protein; midbrain, 1239 ± 225.03; brainstem, 2071.8 ± 80.46; and cerebellum 925.37 ± 148.5. Analysis with one-way ANOVA revealed significant differences between groups in cortex, midbrain, brain stem, and cerebellum ($p < 0.05$). The post-hoc analysis with Student's Newman-Keuls multiple comparisons test further revealed that animals treated with chemicals and stress exhibited a significant decrease compared to controls in cortex and cerebellum ($p < 0.05$). Animals treated with chemicals and stress also exhibited a significant decrease compared to animals treated with stress alone in midbrain and cerebellum ($p < 0.05$). Animals treated with chemicals alone exhibited a significant decrease compared animals treated with stress alone in midbrain ($p< 0.05$). Animals treated with stress alone exhibited a significant decrease compared to controls in cortex and brainstem ($p < 0.05$).

Figure 7 – Cytoarchitecture of liver from a control rat (A), and a rat that was treated with a combination of chemicals and stress (B). Note that following a combined exposure to chemicals and stress, a clear disruption of liver cytoarchitecture is evident (B). This disruption is characterized by sinusoidal dilation, a marked and diffuse steatosis, the presence of microvacuoles, and a mild portal and peri-portal fibrosis containing mononuclear inflammatory cells (arrow). Scale bar, 100 μm.

Figure 8- Alterations in the superficial layer (Layers I - III) of the motor cortex following daily treatment of pyridostigmine bromide (PB) (1.3mg/kg/d, po), DEET (40mg/kg/d, dermal) and permethrin (0.13mg/kg/d, dermal), with and without stress. A1-A4, H& E staining; B1-B4, MAP - 2 immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat that was subjected to stress only. A3 and B3 are examples from a rat treated with chemicals alone. A4 and B4 are examples from a rat treated with both chemicals and stress. A number of degenerating neurons are clearly visible in layers II and III of rats treated with either chemicals alone or chemicals with stress (arrows in A3 and A4). In animals treated with chemicals only, MAP-2 immunoreactivity is significantly reduced (layer III in B3), and dendrites appear wavy. In animals treated with both chemicals with stress, MAP-2 immunoreactivity is dramatically reduced (layer I-III in B4), and the dendrites that are MAP-2 positive have wavy appearance. Scale bar, 100μm.

Figure 9- Changes in the deeper layers (Layers 1V - V) of the motor cortex following daily treatment of pyridostigmine bromide (PB) (1.3mg/kg/d, po), DEET (40mg/kg/d, dermal) and permethrin (0.13mg/kg/d, dermal), with and without stress. A1-A4, H & E staining; B1-B4, MAP-2 immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat subjected to stress only. A3 and B3 are examples from a rat treated with chemicals alone. A4 and B4 are examples from a rat treated with chemicals and subjected to stress. A number of degenerating neurons are clearly visible in layers IV and V of rats treated with chemicals and stress (arrows in A4). In the group that received treatment of only chemicals, the overall MAP-2 immunoreactivity is significantly reduced (layer V in B3), and dendrites appear wavy. In animals treated with both chemicals and stress, the overall MAP-2 immunoreactivity is dramatically reduced (layers IV-V in B4). In addition, a large number of degenerating pyramidal neurons are clearly visible (arrows in A4). Scale bar, 100μm.

Figure 10 – Histograms show the density of surviving (A) and dying (B) neurons /mm2 area of layers III and V of the motor cortex. Values represent means and standard errors (n = 5 per group). Analyses with

one-way ANOVA shows significant differences between groups for both surviving and dying neurons ($p < 0.001$). The post-hoc analysis with Student's Newman-Keuls multiple comparisons test further revealed that animals treated with both chemicals and stress exhibited a significantly decreased number of surviving neurons in both layers III ($p < 0.01$) and V ($p < 0.001$), in comparison to both controls and animals treated with either chemicals or stress. In contrast, animals treated with chemicals alone exhibited a reduced number of neurons compared to controls in only the layer V ($p < 0.05$). Animals treated with both chemicals and stress also exhibit a significantly increased number of dying neurons compared to control animals and animals treated with either stress or chemicals ($p < 0.001$).

Figure 11- Alterations in the dentate gyrus following daily treatment of pyridostigmine bromide (PB) (1.3mg/kg/d, po), DEET (40mg/kg/d, dermal) and permethrin (0.13mg/kg/d, dermal), with and without stress. A1 - A4, H& E staining; B1 - B4, GFAP immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat subjected to stress only. A3 and B3 are examples from a rat treated with chemicals alone. A4 and B4 are examples from a rat treated with both chemicals and stress. A number of degenerating granule cells are clearly visible in the dentate granule cell layer (GCL; A4) of rats treated with both chemicals and stress. Whereas, GFAP immunoreactivity is up-regulated in both animals treated with chemicals alone (B3), and animals treated with chemicals and stress (B4). Increased GFAP immunoreactivity is particularly distinct in dentate molecular layer (ML) and the dentate hilus (DH), Scale bar, 100μm.

Figure 12- Alterations in the CA1 subfield of the hippocampus following daily treatment of pyridostigmine bromide (PB) (1.3mg/kg/d, po), DEET (40mg/kg/d, dermal) and permethrin (0.13mg/kg/d, dermal), with and without stress. A1-A4, H& E staining; B1-B4, GFAP immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat was subjected to stress only. A3 and B3 are examples from a rat treated with chemicals alone. A4 and B4 are examples from a rat treated with both chemicals and stress. A number of degenerating pyramidal neurons are clearly visible in

the stratum pyramidale (SP) of rats treated with both chemicals and stress (arrows in A4). Whereas, GFAP immunoreactive astrocytes are significantly increased in both animals treated with chemicals alone (B3), and animals treated with chemicals and stress (B4). The increased GFAP immunoreactivity is particularly conspicuous in the stratum radiatum. Scale bar, 100μm.

Figure 13- Alterations in the CA3 subfield of the hippocampus following daily treatment of pyridostigmine bromide (PB) (1.3mg/kg/d, po), DEET (40mg/kg/d, dermal) and permethrin (0.13mg/kg/d, dermal), with and without stress. A1-A4, H& E staining; B1-B4, GFAP immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat that was subjected to stress only. A3 and B3 are examples from a rat treated with chemicals alone. A4 and B4 are examples from a rat treated with chemicals and stress. Note degenerating pyramidal neurons in the stratum pyramidale (SP) of rats treated with chemicals and stress. Whereas, GFAP immunoreactive astrocytes exhibit significant increase in both animals treated with chemicals alone (B3), and animals treated with chemicals with stress (B4) in all strata. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Scale bar, 100μm.

Figure 14 – Histograms show the density of surviving (A) and dying (B) neurons per mm2 area of different cell layers of the hippocampal formation. Values represent means and standard errors (n = 5 per group). Analyses with one-way ANOVA revealed significant differences between groups for both surviving neurons (dentate gyrus, $p < 0.001$; CA subfield, $p < 0.05$; and CA3 subfield, $p < 0.001$) and dying neurons (dentate gyrus, $p < 0.05$; CA1 subfield, $p < 0.01$; and CA3 subfield, $p < 0.05$). The post-hoc analysis with Student's Newman-Keuls multiple comparisons test further revealed that animals treated with chemicals and stress exhibited a significant decrease in the number of surviving neurons compared to control animals and animals treated with either chemicals or stress (dentate gyrus, $p < 0.001$; CA1 subfield, $p < 0.05$; CA3 subfield, $p < 0.001$). Further, animals treated with chemicals and stress

exhibited a significantly increased number of dying neurons in all regions of the hippocampal formation compared to both control animals and animals treated with either stress or chemicals alone ($p < 0.05$).

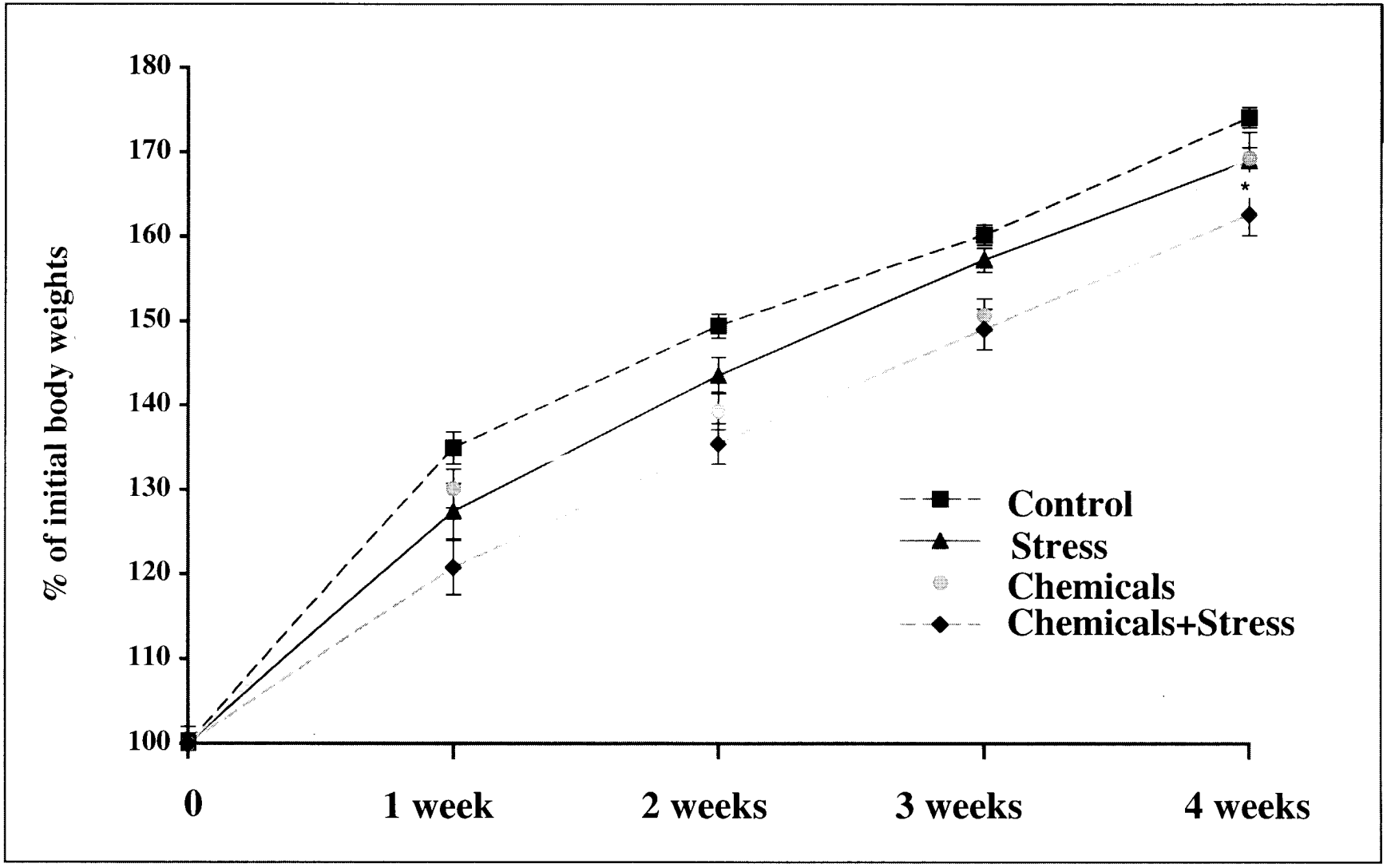

% of initial body weights
180
170
160
150
140
130
120
110
100
0
1 week
2 weeks
3 weeks
4 weeks
*
Control
Stress
Chemicals
Chemicals+Stress

Figure 1
Abdel-Rahman

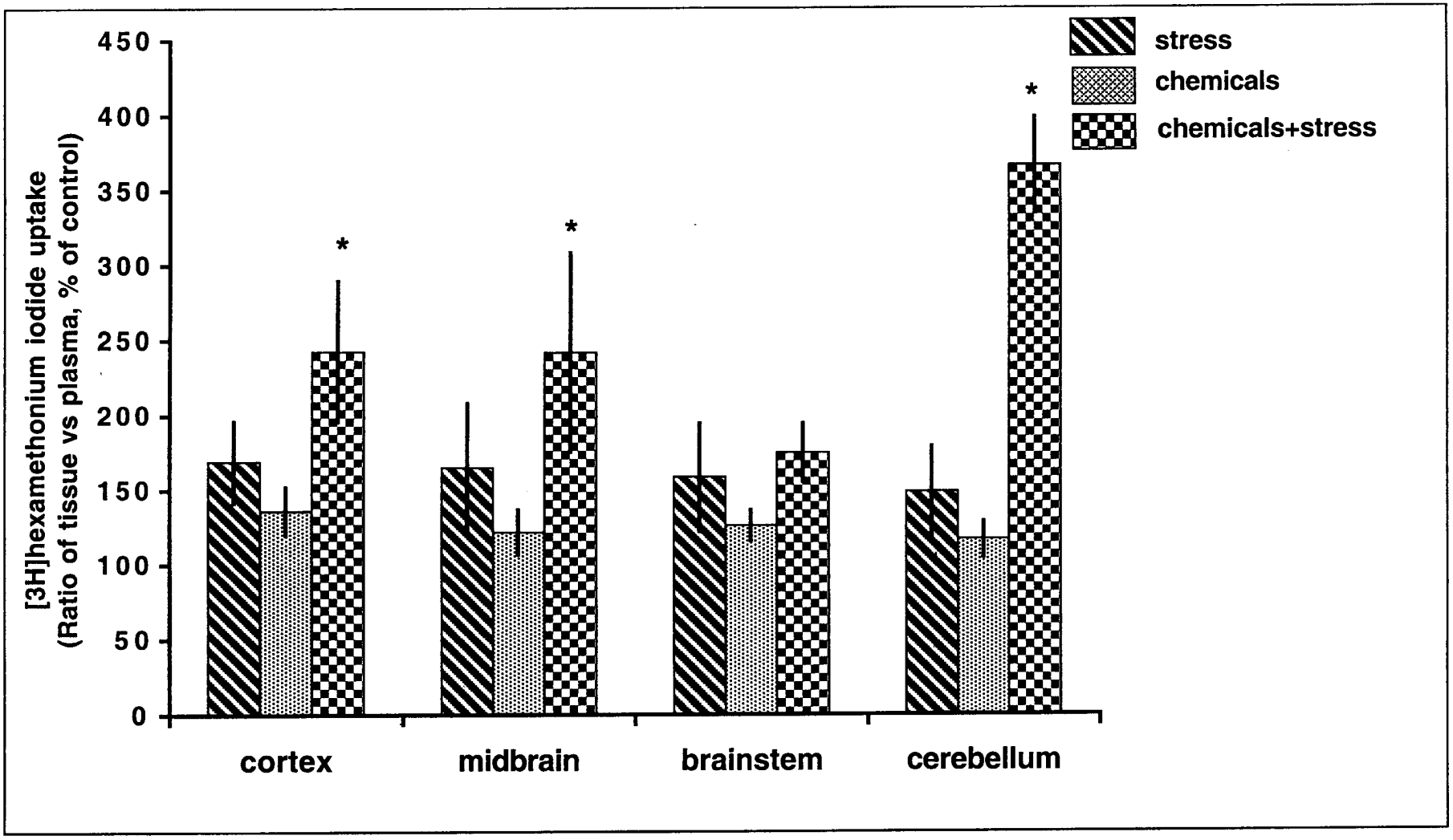
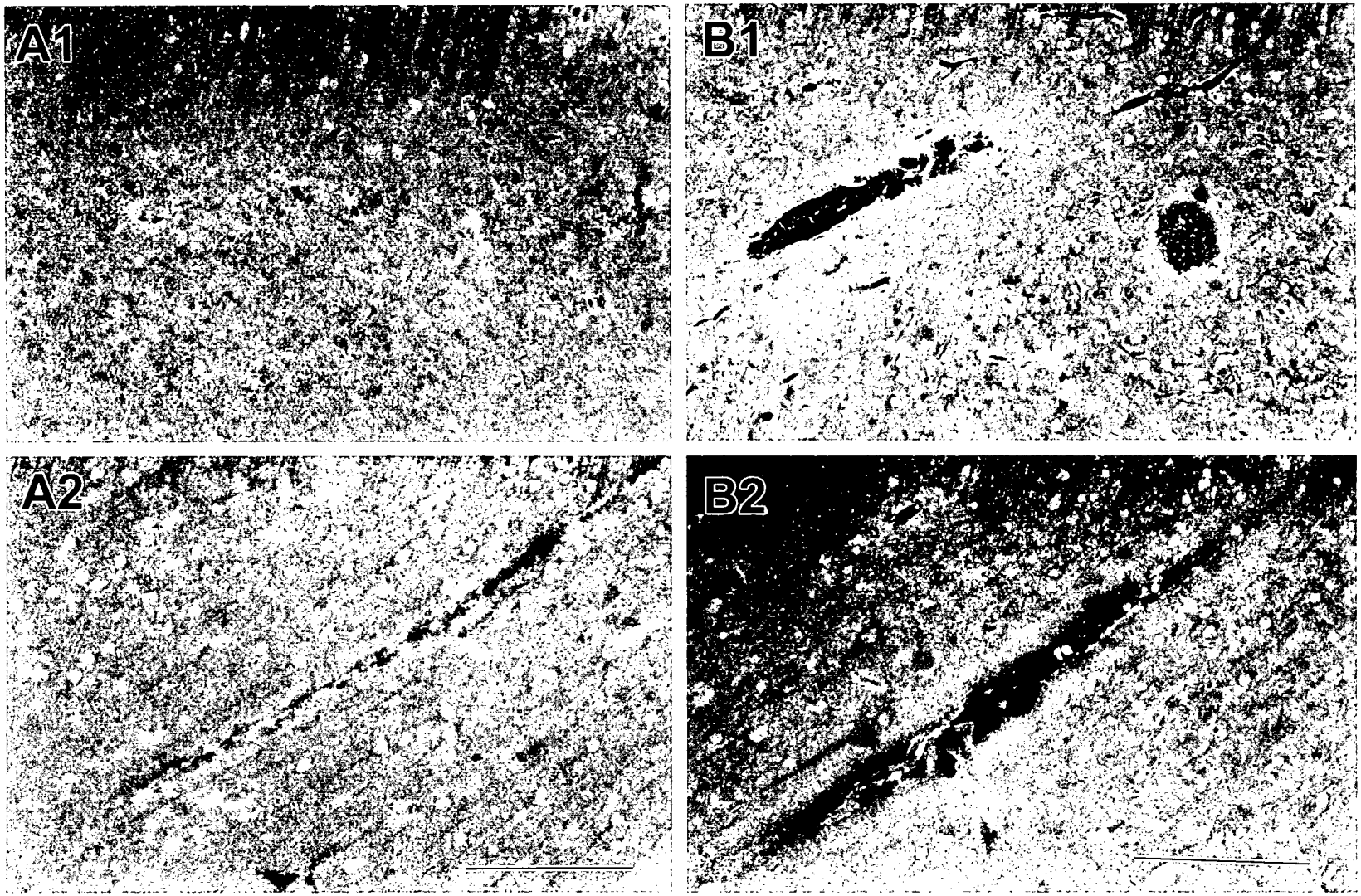

[3H]hexamethonium iodide uptake
(Ratio of tissue vs plasma, % of control)
450
400
350
300
250
200
150
100
50
0
stress
chemicals
chemicals+stress
*
*
*
cortex
midbrain
brainstem
cerebellum

Figure 2
Abdel-Rahman


A1
B1
A2
B2


A
B

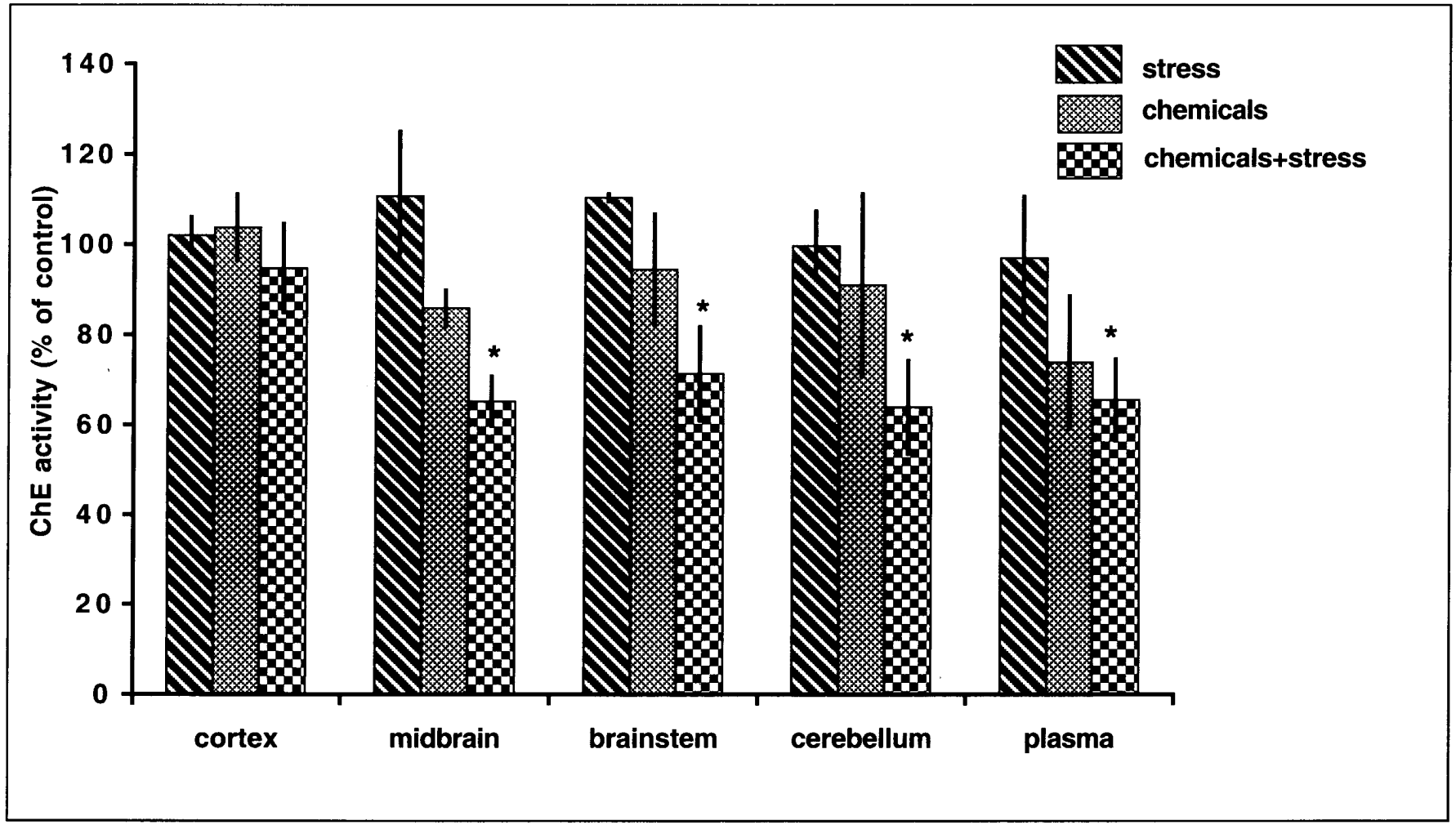

140
120
100
80
60
40
20
0
ChE activity (% of control)
stress
chemicals
chemicals+stress
*
*
*
*
cortex
midbrain
brainstem
cerebellum
plasma

Figure 5
Abdel-Rahman

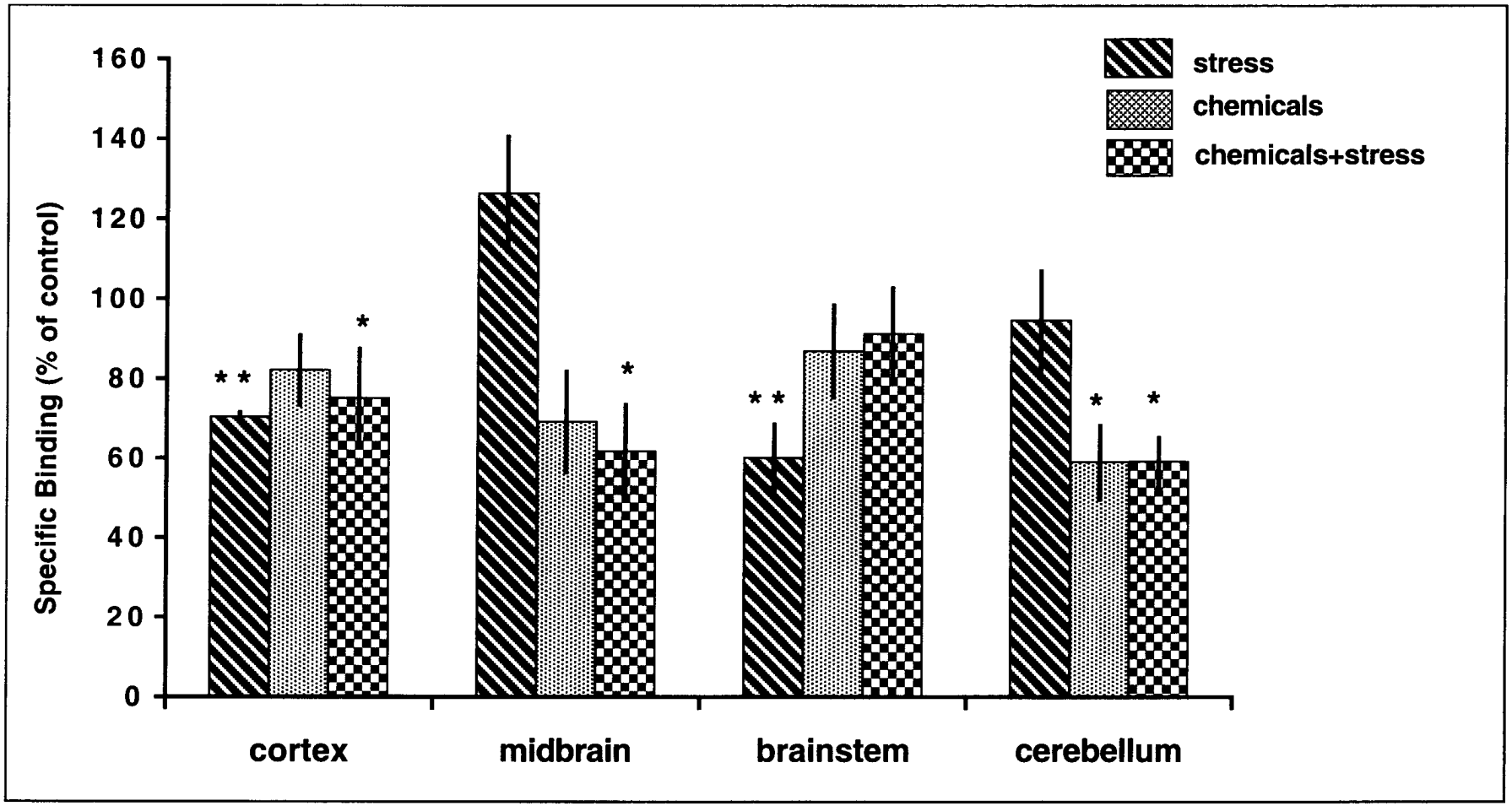
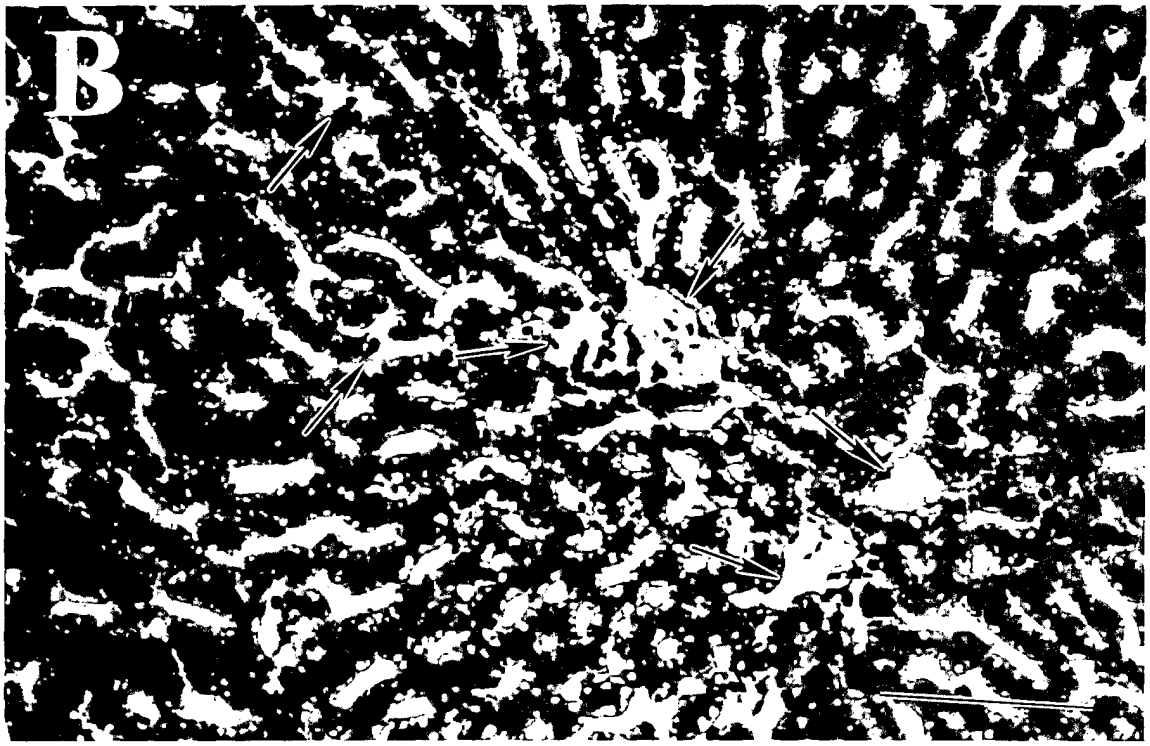
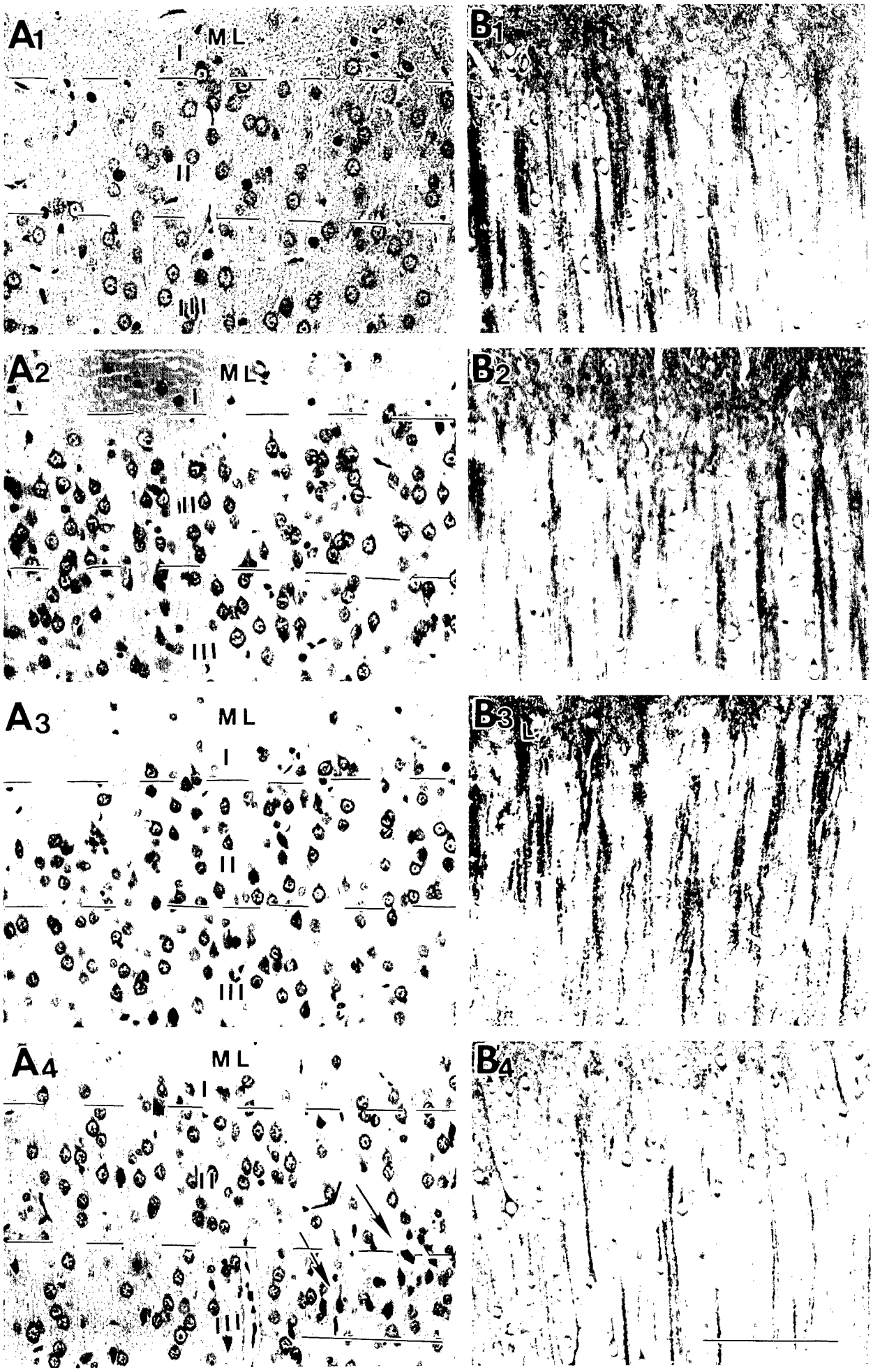
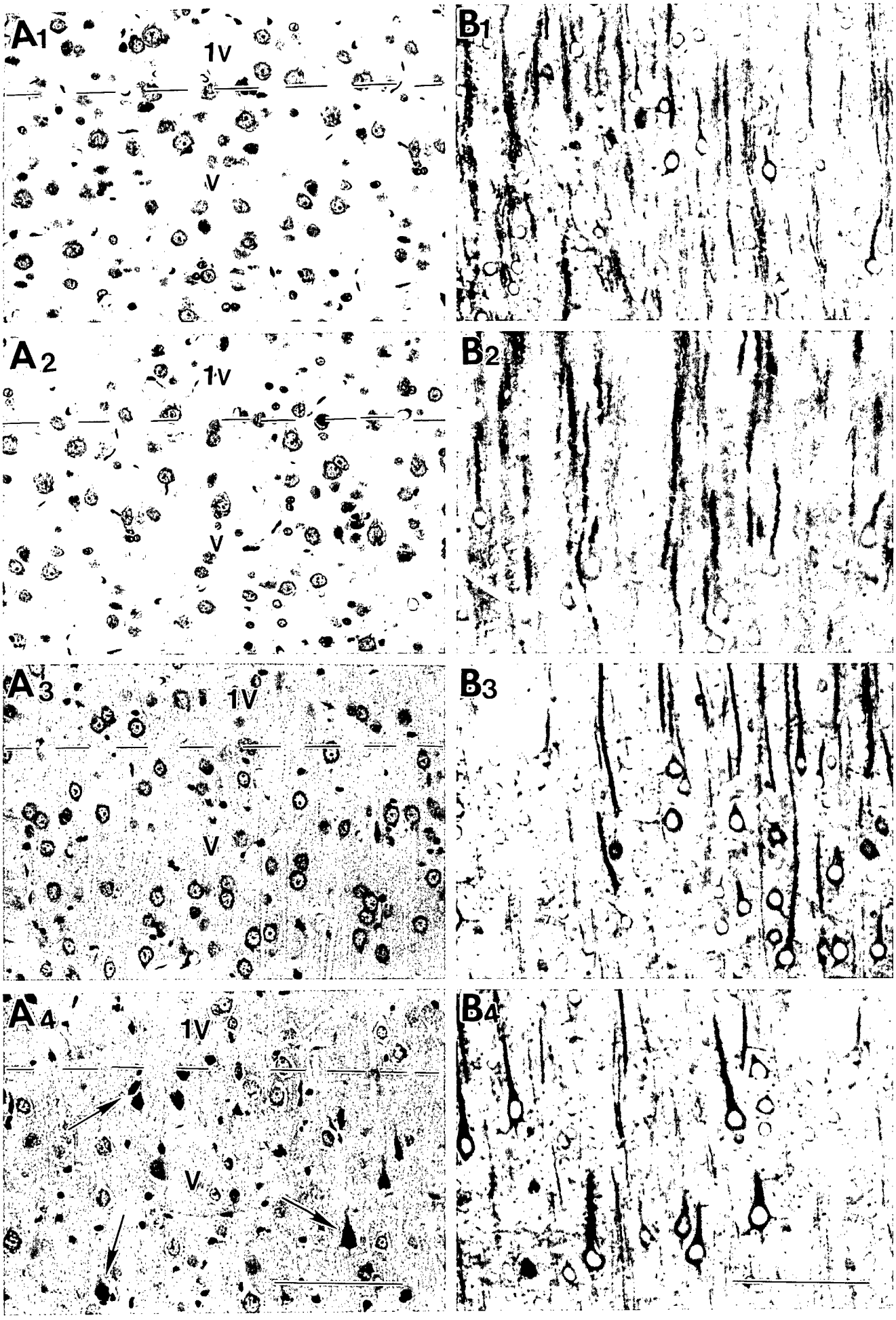

Specific Binding (% of control)
160
140
120
100
80
60
40
20
0
stress
chemicals
chemicals+stress
* *
*
*
* *
*
*
cortex
midbrain
brainstem
cerebellum

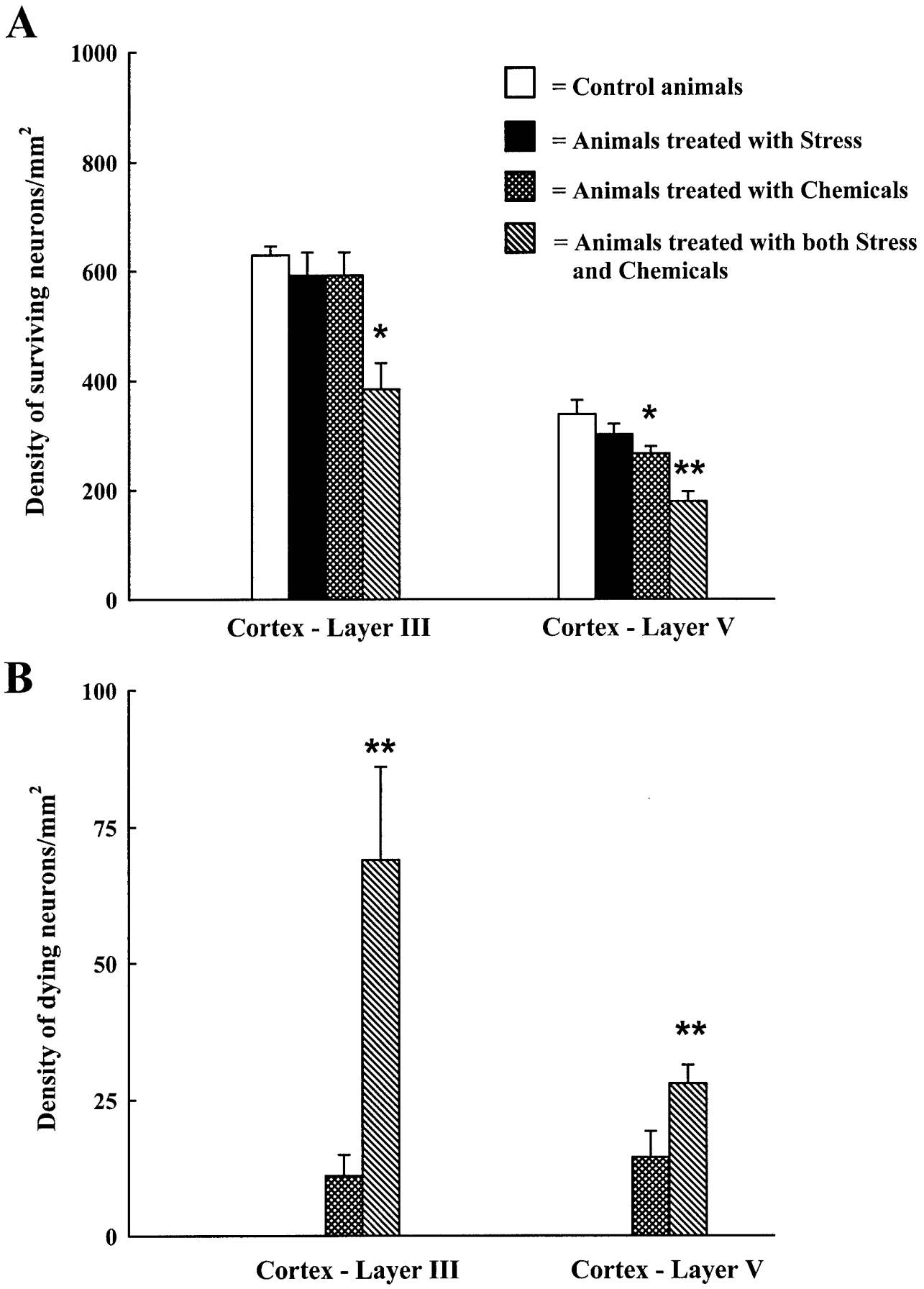
Figure 6
Abdel-Rahman

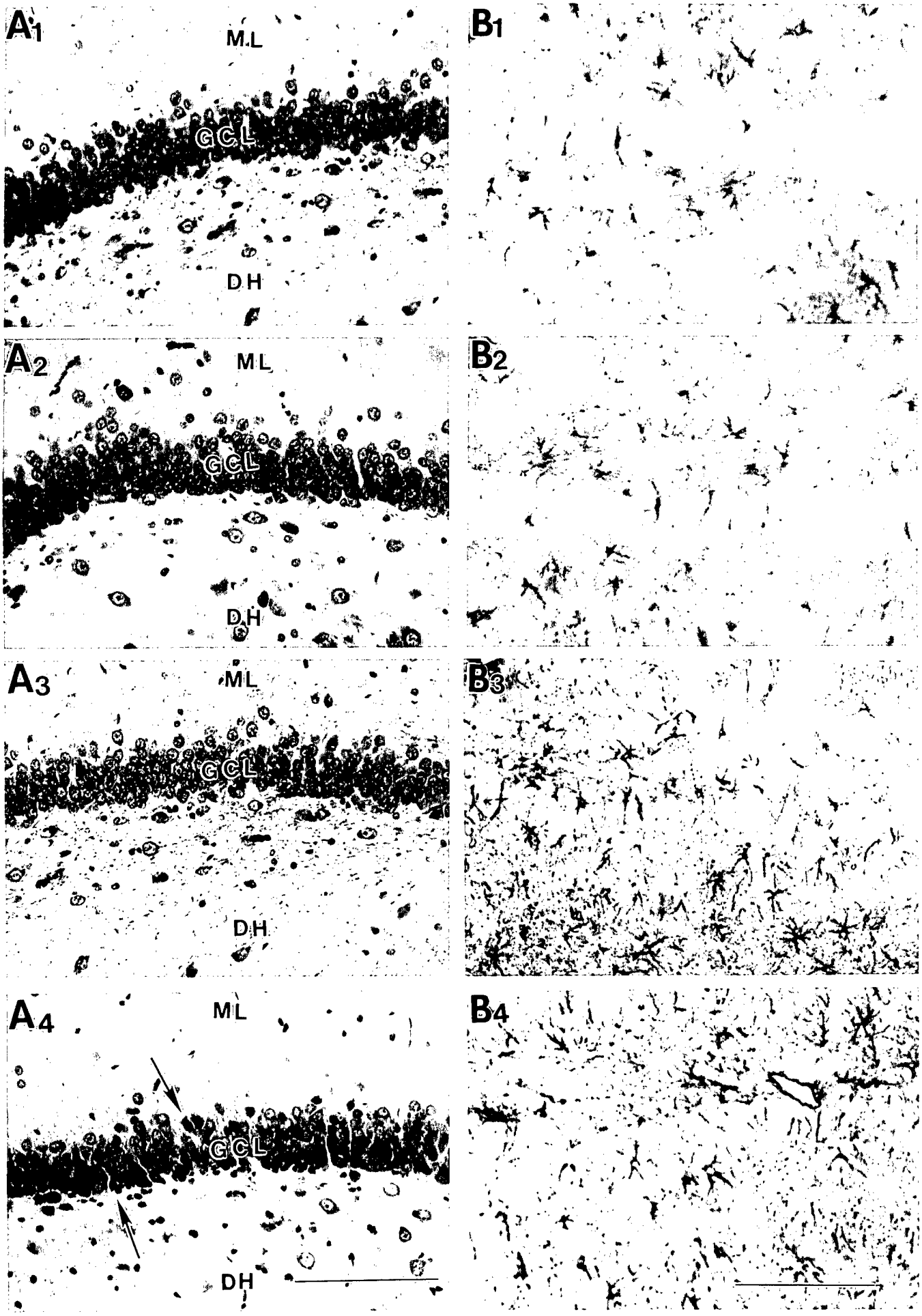

A

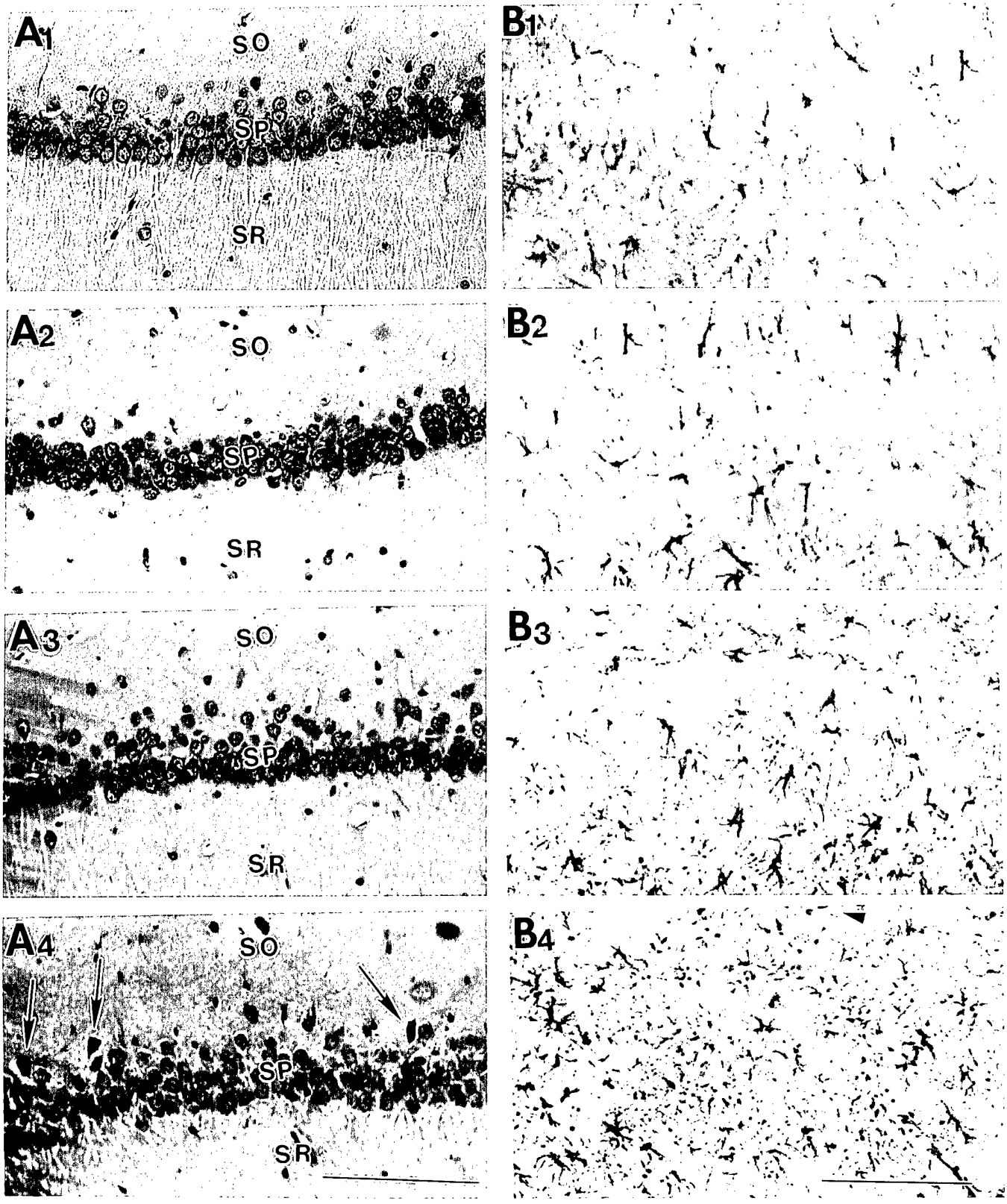

B

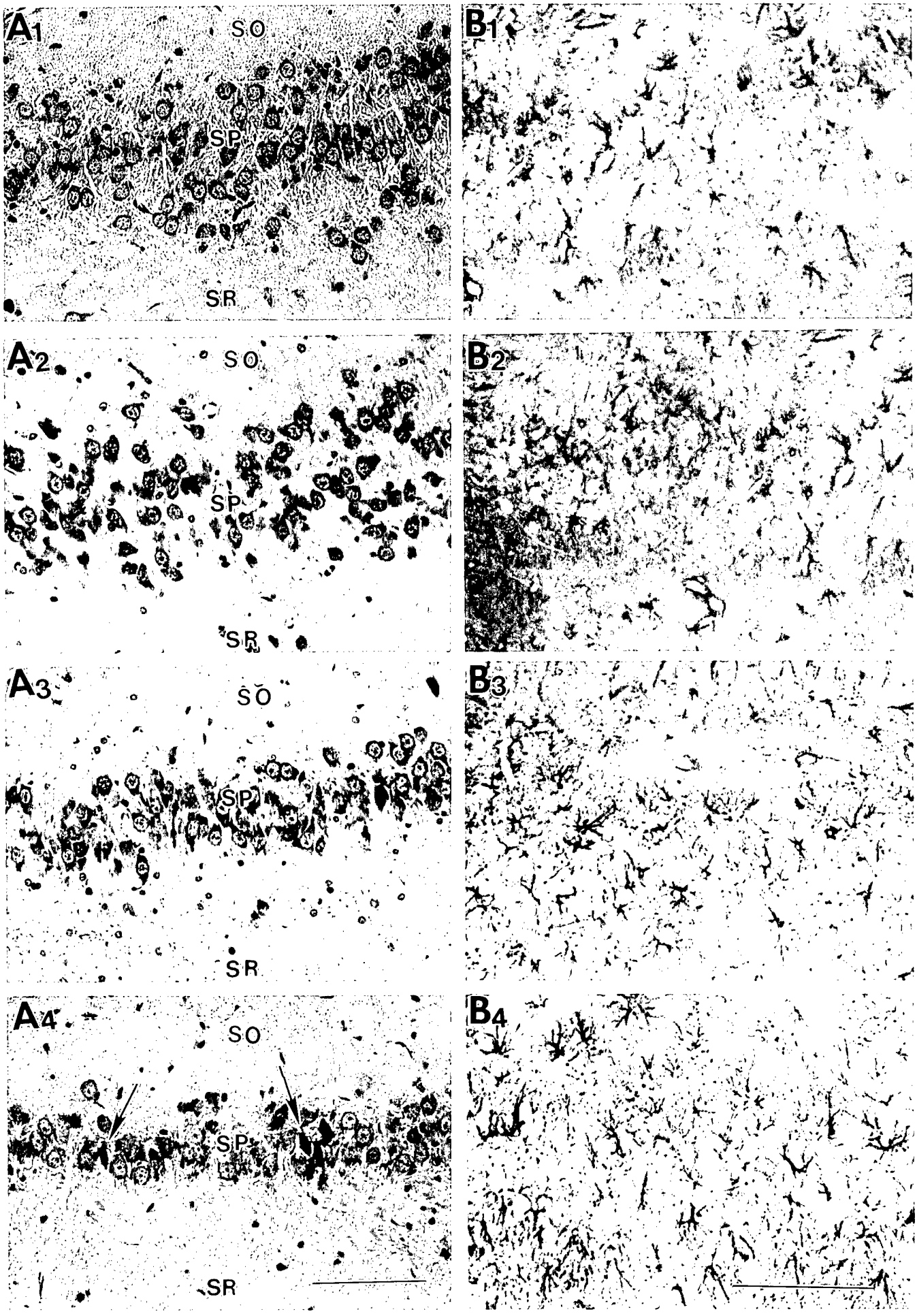

A1
ML
I
II
III
B1
A2
ML
I
III
B2
A3
ML
I
II
III
B3
A4
ML
I
II
III
B4

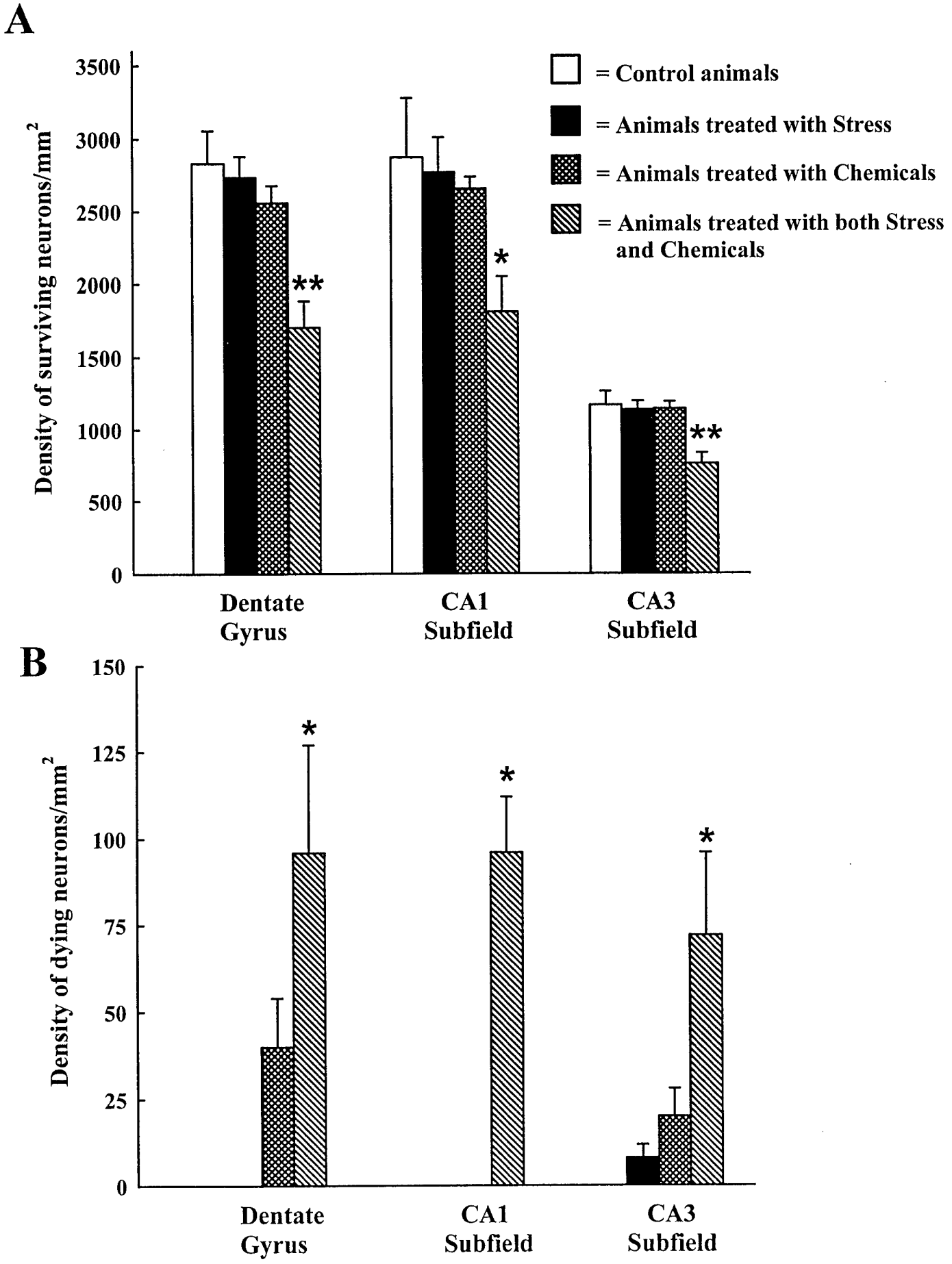

A1
1V
V
B1
A2
1V
V
B2
A3
1V
V
B3
A4
1V
V
B4


A
1000
800
600
400
200
0
Density of surviving neurons/mm2
= Control animals
= Animals treated with Stress
= Animals treated with Chemicals
= Animals treated with both Stress and Chemicals
*
*
**
Cortex - Layer III
Cortex - Layer V
B
100
75
50
25
0
Density of dying neurons/mm2
**
**
Cortex - Layer III
Cortex - Layer V


A1
ML
GCL
DH
B1
A2
ML
GCL
DH
B2
A3
ML
GCL
DH
B3
A4
ML
GCL
DH
B4


A1
SO
SP
SR
B1
A2
SO
SP
SR
B2
A3
SO
SP
SR
B3
A4
SO
SP
SR
B4


A1
SO
SP
SR
B1
A2
SO
SP
SR
B2
A3
SO
SP
SR
B3
A4
SO
SP
SR
B4


A
3500
3000
2500
2000
1500
1000
500
0
Density of surviving neurons/mm2
= Control animals
= Animals treated with Stress
= Animals treated with Chemicals
= Animals treated with both Stress and Chemicals
**
*
**
Dentate Gyrus
CA1 Subfield
CA3 Subfield
B
150
125
100
75
50
25
0
Density of dying neurons/mm2
*
*
*
Dentate Gyrus
CA1 Subfield
CA3 Subfield

Appendix 3
DAMD# 17-99-1-9020
Mohamed B. Abou-Donia

PHARMACOKINETS INTERATION BETWEEN DEET (*N,N*-DIETHYL *m*-TOLUMAMIDE) AND PERMETHRIN FOLLOWING DERMAL ADMINISTRATION IN RATS.

Abstract

The pharmacokinetic interactions between a single dermal dose of the insecticide permethrin (3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl) methyl ester) and the insect repellent DEET (*N,N*-diethyl-*m*-toluamide) has been investigated in rats. Rats were dosed with either 1.3 mg/kg (0.325 mg/1 cm^2 of skin area) of permethrin, 400 mg/kg (100 mg/ 1cm^2 of skin area) of DEET, alone or with both agents at these doses. Five rats were sacrificed at each time interval of 0.5, 1, 2, 4, 8, 24, 48, and 72 h after dosing. Plasma, liver, kidney, brain, testes, and urine collected and analyzed for permethrin, DEET, and their metabolites by high performance liquid chromatography (HPLC). In rats treated with a single agent, 0.5 and 72 h after dosing, the application site retained 45%, and 0.05% of DEET, and 62% and 4% of permethrin, respectively. Distribution of permethrin in tissues was slower compared to DEET. At 8 hr after dosing, maximum concentrations of permethrin were 76 and 185 ng/g in liver and kidney, respectively. Permethrin metabolites *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were identified in plasma, liver and kidney 24 h after administration. DEET and its metabolites *m*-toluamide and *m*-toluic acid were detected in plasma and tissues within 1 h of dosing. The time concentration curves of DEET and permethrin in plasma was fitted to a one compartment pharmacokinetic model with a terminal half-life of elimination from plasma of 32.6 h and 22.9 h for DEET and permethrin, respectively. DEET and its metabolites *m*-toluamide and *m*-toluic acid were detected in urine samples before hydrolysis. Sequential enzymatic hydrolysis of urine samples showed that *m*-toluamyl glucuronide and *m*-toluamyl sulfate conjugates were excreted in urine. Neither permethrin nor its metabolites *m*-phenoxybenzoic acid and *m*-

phenoxybenzyl alcohol were detected in urine samples. Sequential enzymatic hydrolysis of urine samples yielded *m*-phenoxybenzyl alcohol, indicating that *m*-phenoxybenzlyl glucuronide and *m*-phenoxybenzlyl sulfate were excreted in urine following permethrin administration. Hot acid hydrolysis of urine samples yielded unidentified metabolites of DEET and permethrin.

Combined administration of both compounds increased AUC_{plasma} of DEET compared to AUC_{plasma} of DEET when applied alone. No effect on AUC_{plasma} of permethrin in the presence of DEET has been detected. There was no significant effect on absorption or excretion of either compound following dermal application of combination of DEET and permethrin compared to individual application of each compound. The results showed that combined exposure to permethrin and DEET could prolong presence of DEET in the circulation system, thus reduce its rate of elimination.

Key words: DEET, permethrin, Gulf War syndrome, combined exposure.

Introduction

Permethrin is a pyrethroid insecticide effective against mites and head lice (Burgess et al., 1992; Fraser, 1994; Miller, 1989). DEET is a widely used insect repellent used against mosquitoes and other biting insects (USEPA, 1998; Robinson and Cherniak, 1986; Brown and Hebert, 1997). Both compounds were used during the Gulf War for pest control (Young and Evans, 1998). Permethrin modifies sodium channel to open longer during a depolarization pulse (Narahashi, 1985). Previous studies showed that DEET has direct effect on the nervous system in laboratory animals resulting in spongiform myelinopathy in the brain stem with signs including ataxia, seizures, coma, and death (Verschoyle et al, 1990). Furthermore, extensive and repeated topical application of DEET resulted in human poisoning including two deaths (Edwards and Johnson, 1987; Roland et al., 1985). Combined exposure to permethrin and DEET enhanced neurotoxicity in hens (Abou-Donia et al, 1996), modified the blood brain barrier in rats (Abou-Donia et al, 2001a, 2001b), caused oxidative stress in rats (Abu-Qare and Abou-Donia, 2000b, 2001b, 2001c; Abu-Qare et al, 2001), increased mortality in rats (McCain et al, 1998), and caused behavioral alterations in male rats (Abou-Donia et al., 2001a; Hoy et al, 2000a, 2000b). Published reports implicated exposure to permethrin and DEET in Gulf War illnesses (Abou-Donia et al., 1996; Haley and Kurt, 1997; Olsan et al., 1998; Kurt, 1998; Shen, 1998; Wilson et al., 1998; Jamal, 1998; Haley et al., 1999). Metabolism of permethrin has been examined following oral or *i.v* dose in rats (Abu-Qare and Abou-Donia, 2000a; Anadon et al, 1991). Disposition and metabolism of DEET has been studied in vivo following dermal application to human volunteers (Abu-Qare and Abou-Donia, 2000a; 2001c; Seliem et al., 1995; Blomquist

and Thorsell, 1977), in rats (Schoeing et al., 1996); in mice (Blomquist and Thorsell, 1977), in cattle (Taylor et al., 1994) and after topical application in beagle dogs (Qiu et al., 1997). DEET metabolism involved *N*-dealkylation, ring hydroxylation and ring dealkylation following *in vitro* incubation with rat liver microsomes (Constantino and Iley, 1999; Taylor, 1986).

This study reports on results of absorption, disposition, metabolism and excretion of single dermal doses of permethrin, or DEET, and a single dermal dose of both agents in rats.

Materials and methods

Chemicals

Permethrin (99%, (3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) was purchased from Chem Service Inc. (West Chester, PA). *m*-Phenoxybenzyl alcohol, *m*-phenoxybenzoic acid, *β*-glucuronidase from bovine liver type B-1, and sulfatase from *limpets* type V were obtained from Sigma Chemical Co., (St. Louis, MO). DEET (≥97% *N,N*-diethyl-*m*-toluamide) was obtained from Aldrich Chemical Co, Inc. (Milwaukee, WI). *m*-Toluamide, and *m*-toluic acid were purchased from Fisher Scientific (Pittsburgh, PA). C_{18} Sep-Pak^R cartridges were obtained from Waters Corporation (Waters Corporation, Milford, MA). Water and acetonitrile (HPLC grade) were obtained from Mallinckrodt Baker, Inc. (Paris, KY).

HPLC System

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector and a Waters 2487 dual λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2 cm × 4.0 mm, 5μm (Supelco Park, Bellefonte, PA), and a reversed-phase C_{18} column μBondapak C_{18} 10 μm, 3.9 × 300 mm were used, (Waters Corporation, Milford, MA) were used.

Chromatographic conditions

The mobile phase was water (adjusted to pH 3.0 using 0.1N acetic acid): acetonitrile gradient at flow rate ranging between 1.0-1.7 ml/min. The gradient started at 1%

acetonitrile, increased to 25% acetonitrile at 3.6 min, then to 45% acetonitrile at 6 min, and up to 85% acetonitrile at 11 min. The system returned to 1% acetonitrile at 14 min and was kept under these conditions for 3 min to re-equilibrate. Total run time was 17 min. The analytes were monitored by UV detection at 210 nm for permethrin, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, and 230 nm for DEET, *m*-toluamide, and *m*-toluic acid. The chromatographic analysis was performed at ambient temperature.

Calibration procedures and detection limits

Five different calibration standards of a mixture of permethrin, DEET, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were prepared in acetonitrile. Their concentrations ranged from 100-1000 ng/ml following a method developed and validated in our laboratory (Abu-Qare and Abou-Donia, 2000a). Linear calibration curves were obtained by plotting the peak areas of the individual chemicals as a function of concentration. The standard curves were used to determine recovery of the chemicals from plasma and tissue samples. Limits of detection were determined at the lowest concentration that can be detected, taking into consideration a 1:3 baseline noise: calibration point ratio.

Recovery of permethrin, DEET and metabolites

Plasma, urine and tissue samples from control animals were spiked with selected concentration ranging between 100-1000 ng/ml of each permethrin, DEET and metabolites (Abu-Qare and Abou-Donia, 2000a). The samples were extracted and

cleaned up as described under sample preparation. Percentage recoveries of each compound were determined using the calibration curves as described above.

Experimental animals

Sprague Dawley rats (250-300 g) were purchased from Zivic Miller (Zelienople, PA). The animals were kept in a 12-h light/dark cycle (temperature 21-23°C) and were provided with a free supply of feed (Rodent Laboratory Chow, Ralston Purine Co., (St. Louis, MO) as well as tap water. Animal care was conducted according to institutional guidelines.

Animal treatment

Permethrin and DEET were dissolved in 70% ethanol. A single dose of 1.3 mg/kg of permethrin or 400 mg/kg of DEET, or both doses in combination (DEET followed by permethrin) were applied with a micropipette (1 ml/kg) to an unprotected 1 cm^2 area of pre-clipped skin on the back of each rat. The application area was not protected to resemble real-life situations. A group of five animals was used for each time point. The doses were selected to represent real-life exposure levels (Abou-Donia et al., 1996; 2001a. 2001b). Control animals were treated with equal volume of 70% ethanol either to resemble single application (250 µl) or combined application (500 µl) and kept under similar conditions as treated rats.

Animals handling

Rats were placed into metabolic cages after dosing to facilitate urine collection and five rats per time point were anesthetized with halothane (Halothane Laboratories, River Edge, PA) at intervals of 0.5, 1, 2, 4, 8, 24, 48, and 72 hr after dosing and exsanguinated by cardiac puncture into heparinized syringes. Samples of liver, kidney, brain and testes were collected from each animal and portion of the blood volumes were separated into plasma samples by centrifugation at 2400 rpm for 10 min at 5°C. Plasma and tissue samples were stored at -70°C until analysis

Sample preparation

Plasma (0.5 ml), and urine (1.0 ml) sample, or 0.5 g of liver, kidney, brain, and testes were each mixed or homogenized with a 2 ml of (1:1) of acetonitrile and methanol, centrifuged at 1000g for 5 min, and the supernatant was removed. Disposable C_{18} Sep-Pak Vac 3cc (500mg) cartridges were conditioned with 3 ml of acetonitrile, then equilibrated using 3 ml of water prior to use. The supernatant was loaded into the disposable cartridges, washed by 3.0 ml of water, followed by elution with 2 ml of methanol, and 2 ml of acetonitrile. The elution volume was reduced to 500 µl (0.5 ml) in a test tube rack using a gentle stream of nitrogen, prior to analysis by HPLC.

Urine Analysis

Non-conjugates

Each urine sample (1 ml) was acidified using 0.1N phosphoric acid (pH 4) and passed through Sep pack cartridges as described above. The contents were analyzed by using HPLC to determine parent compound and non-conjugated metabolites.

Conjugates

(a) Enzymatic hydrolysis

i) Glucuronides. β-glucuronidase from bovine liver type B-1 was added to urine residues in 0.2 M sodium acetate buffer (pH 4.5), incubated for 18 hours at 37°C, then the urine incubate was analyzed as previously described under sample preparation,

(ii) Sulfates. The remaining urine residue was incubated for 24 hours at 37°C with sulfatase from *limpets* type V in 0.2 M sodium acetate buffer (pH 4.5). The residue was analyzed as described above under sample preparation.

(b) Hot acid hydrolysis

Another 1.0 ml urine sample was adjusted by 1 N sulfuric acid to (pH 2.5), heated for 2 hours at 80°C, and analyzed as described above.

Kinetics analysis

The kinetic analysis of permethrin, DEET and metabolites in plasma and tissues were performed using WinNonlin Program (Pharsight Corporation, Mountain View, CA). The

terminal half-life of permethrin, DEET and their metabolites was calculated from the elimination rate constant K_{10}, that was obtained by linear regression of the terminal linear exponential decline in concentration. The total area under the compound concentration *vs.* time curves for plasma, AUC_{plasma}, or brain AUC_{brain} was calculated by the trapezoidal rule and extrapolated to infinity by using the last data point and the respective terminal linear experimental decline.

Statistical analysis

The pharamcokinetics parameters were subjected to analysis of variance (ANOVA) to determine if the difference between treated and control samples is significance using a GraphPad Prism program for Windows (GraphPad Software, Inc., San Diego, CA) t. A value of $P<0.05$ was considered statistically significant.

RESULTS

Clinical observation

A single dermal dose of 1.3 mg/kg of permethrin, or a single dermal dose of 400 mg/kg of DEET, or both agents at these doses in combination did not produce observable toxic effect in rats, based on gross examination without morophological or physiological studies carried out. Both control and treated animals consumed comparable amounts of feed and water. There was no difference in weight, size, shape, or color of various tissues of treated animals compared with tissues of control rats.

Limits of detection and recovery of permethrin, DEET, and metabolites

Average percentage recoveries of permethrin, DEET, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid from plasma were 82.3 ± 8.2 71.1 ± 11.2, 73.6 ± 10.1, 79.3 ± 8.5, 84.3 ± 12.0, and 82.6 ± 6.1, respectively. Their limits of detection were 30, 50, 50, 80, 20, and 30 ng/ml, respectively.

Dissipation of permethrin and DEET from the application site

Permethrin and DEET were absorbed at different rate from rat skin following a single dermal dose. The application site retained 62% and 4% of permethrin, and 45% and 0.05% of DEET after 0.5 hr and 72 hr following application, respectively. Rate of dissipation of both compounds was rapid during the early time after application, then gradually decreased (Table 1)

DEET and permethrin in plasma and tissues

Tables 2 and 3 list the concentrations of permethrin and DEET (ng /g fresh tissue or mg/ ml plasma) at different time intervals following a single dermal dose of both chemicals in rats. Permethrin, DEET and their metabolites were distributed and detected in analyzed tissues. Permethrin was found in the kidney, liver, brain and plasma. Maximum concentration of DEET was also detected in the analyzed tissues in the following order kidney > liver > plasma > brain > testes. Following administration of a combined dose of permethrin and DEET, higher amount of DEET was detected in plasma (Table 4), while there was no significant change in the pattern of distribution and amount of permethrin and its metabolites when it was concurrently applied with DEET (Table 5).

Metabolism of permethrin and DEET

Metabolites of permethrin and DEET were analyzed using high performance liquid chromatography (HPLC) (Fig 1). *m*-Phenoxybenzyl alcohol, a metabolite of permethrin, was detected in liver, while *m*-phenoxybenzoic acid was detected in liver, kidney and plasma over the time course of analysis. DEET metabolites *m*-toluic acid and *m*-toluamide were found in all analyzed tissues and plasma (Tables 2 and 3). Proposed metabolic pathways of permethrin and DEET are shown in Figures 2 and 3.

Urinary excretion

Urinary excretion was rapid following dermal application of DEET. DEET and its metabolite *m*-toluamide were identified in urine shortly after application. Following enzymatic hydrolysis with glucuronidase, the level of the DEET metabolite *m*-toluamide

was increased, also the metabolite *m*-toluic acid was detected, indicating that *m*-toluamyl and toluyl glucuronide conjugates were excreted in the urine. Incubations of urine samples with sulfatase resulted in a significant increase in the concentrations of both metabolites ($p<0.0$), a further indication of existence of *m*-toluamyl sulfate and *m*-tolyl sulfate conjugates (Fig.4). Urinary excretion of permethrin metabolites was slower compared to DEET. A similar trend was detected following incubation of permethrin urinary excretion with glucuronidase and sulfatase. *m*-Phenoxybenzyl alcohol was detected (Fig.4). Acid hydrolysis of urine samples produced unidentified metabolites for both DEET and permethrin.

Pharmacokinetics profiles of permethrin, DEET, and metabolites

Permethrin and DEET disappeared mono-exponentially from plasma following a single dermal dose in rats (Tables 6 and 7). The time concentration curves of permethrin and DEET in plasma were fitted to one compartment model (Figures 5 and 6). The half-life of elimination of permethrin and DEET from rat plasma was 22.9 and 32.6 h, respectively.

The pharmacokinetic parameters of permethrin, DEET and their metabolites are listed in Table 6 and 7.

Pharmacokinetics interactions

Concurrent administration of permethrin and DEET had no significant effect on rate of absorption of either compound (Table 1). Pharmacokinetic profiles of permethrin, and DEET following combined administration revealed that permethrin increased DEET

AUC_{plasma} (Figure 7 and Table 6) ($P<0.05$). No significant effect has been detected on AUC_{brain} of DEET following combined administration of DEET and permethrin ($P<0.05$). DEET half-life in the brain was 22.9 h and 23.5 h following administration, alone or in combination with permethrin, respectively. There was no significant effect on permethrin pharmacokinetic profiles (plasma or brain) following concurrent application with DEET (Figure 8 and Table 7) ($P>0.05$).

Discussion

A single dermal dose of 400 mg/kg of DEET was rapidly absorbed and distributed in tissues after administration in rats. These results are in agreement with previous reports on absorption and disposition of DEET in rats (Schoenig et al., 1996), in mice (Blomquist and Thorsell, 1977), in beagle dogs (Qiu et al., 1997), and following dermal application to human volunteers (Seliem et al., 1995). Furthermore, DEET has been shown to rapidly cross the dermal barrier following topical application in rats (Windhenser et al., 1982). In the present study DEET was applied in ethanol. Its pattern of absorption could be different if applied in other carrier vehicles. Qiu et al. (1998) reported that *in vitro* skin permeation, the vehicle showed marked effect on rat skin penetration of DEET. Absorption of DEET was significantly correlated to the type of commercial formulation following application in rats (Stinecipher and Shah, 1997). Our findings showed that while 11% of the applied dose of DEET was retained by the application site 24 h after application, more than 99% of the applied dose of DEET disappeared from the application site 72 h after application. These data are consistent with those reported by Schoeing (1996), that 16% of the dose was retained by the application site 36 hr after dermal dose of DEET in human volunteers and by Taylor et al. (1994) that 72.9% of DEET was absorbed into the systemic circulation following dermal single dose in cattle.

DEET was distributed rapidly in the tissues analyzed with a maximum concentration found within 4-8 hr after application; liver>kidney>plasma>brain>testes. In previous study, Blomquist and Thorsell (1977) reported that blood, kidney and liver

had high concentration of radioactivity after cutaneous application of ^{14}C-*N,N*-diethyl-*m*-toluamide in mice.

Absorption of permethrin from the application site was slower compared to DEET, possibly due to differences between the compounds in their molecular weights and physical properties such as lipid solubility. Only 4% of administered dose of permethrin was retained by the application site after 72 h. This is in agreement with Anadon et al (1991) who reported that permethrin was absorbed slowly following an oral dose in rats. In addition to its absorption by rat skin, permethrin and DEET could be dissipated from rat skin through other pathways such as volatilization, or through contact with surfaces of metabolic cages.

Permethrin and metabolites were detected in tissues and plasma within 0.5-24 hr after application. The findings are consistent with the results of Anadon et al (1991) who reported that permethrin was distributed in tissues and accumulated in the nervous system following an oral dose of 460 mg/kg in rats.

Figures 2 and 3 present a suggested scheme for the metabolism of dermal dose of DEET and permethrin. DEET metabolites *m*-toluamide and *m*-toluic acid were detected in most of analyzed tissues. DEET underwent metabolic transformation mediated by esterases and cytochrome p450 enzymes, resulted in formation of metabolites of *m*-toluic acid and *m*-toluamide. This is in agreement with previous reports following dermal dose of DEET in rats and dogs (Schoenig et al, 1996; Qiu et al, 1997), and in accordance with previous studies reported that DEET metabolism mediated by *N*-dealkylation, ring

hydroxylation and ring dealkylation following *in vitro* incubation with rat liver microsomes (Constantino and Iley, 1999; Taylor, 1986). These findings are also consistent with our recent results obtained following *in vivo* and *in vitro* incubation of DEET with human liver microsomes (Abu-Qare and Abou-Donia, 2001b; 2001d).

In this study, DEET metabolite *m*-toluic acid was detected in plasma 1 hr after application indicates the rapid hydrolysis of DEET *in vivo*. Permethrin and its metabolites *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid were also detected at 2 h after application in plasma and tissues, showing the role of esterases in its metabolism. In previous results, permethrin metabolites *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were detected in plasma and tissues up to 48 h after an oral dose in rats (Anadon et al, 1991). The rapid metabolism of permethrin reported in this study indicates its rapid hydrolytic cleavage as an initial phase of metabolism. Rapid hydrolysis of permethrin was catalyzed by butyrylcholinesterases following *in vitro* incubation with human plasma, where addition of butyrlcholinesterase inhibitor Iso-OMPA significantly inhibited permethrin metabolism *in vitro* (Abu-Qare and Abou-Donia, 2001d). Furthermore, permethrin metabolism proceeded through oxidation and the formation of *m*-phenoxybenzoic acid. This is in accordance with a report that permethrin oxidative pathway is mediated by cytochrome P450 enzymes, thus more toxicity has been detected when an oxidase inhibitor PB (piperonyl butoxide) was applied with permethrin in rats (Vulule et al, 1999). In other study, permethrin induced cytochrome P-4502B in rat liver (Koska et al, 1997).

The present results suggest that the pharmacokinetics analysis of DEET following a single dermal dose of 400 mg/kg would require a one-compartment open-model system. Elimination half-life of DEET was 32 h, while T_{max} in plasma was 2.1 h and C_{max} was 2103 ng/ml. It seems that dose, and route of administration of DEET were factors in the determination of its pharmacokinetic profile and its rate and pattern of elimination. Qiu et al. (1997) reported that the pharmacokinetic profile of DEET was bei-exponential with an elimination half-life of 2.7 h, after dermal dose in Beagle dogs, where its plasma concentration peaked 1-2 h after dosing. Furthermore Hoy et al. (2000) reported that blood serum concentration of DEET was 12347 ng/ml following an i.p injection of 500 mg/kg in male rats. In another study, DEET's elimination half-life was 200.2 min following a dermal dose in human volunteers (Schoeing, 1996). Following its administration, only small amount of the intact DEET was excreted in the urine. Majority of the urinary excretion following a single dermal dose of DEET and permethrin was in the form of conjugated metabolites. This was confirmed following sequential enzymatic hydrolysis with glucuronidase and sulfatase.

Our results also suggested that permethrin is also fitted to one compartment pharmacokinetic model, with half-life of elimination from plasma of 22.9 h. This is in accordance with Anadon et al (1991) who suggested one compartment model for the plasma profile of permethrin following oral dose of 460 mg/kg or *i.v* dose of 46 mg/kg in rats, where elimination half-life of permethrin was from rat plasma following an oral dose was 12.37 hr.

The results show that dermal administration of a combined dose of DEET and permethrin resulted in significant increase in the AUC_{plasma} of DEET in rats, with a slight increase in its half-life of elimination. Permethrin appears to inhibit or compete for DEET metabolism in plasma *in vivo*. In contrast to the effect of permethrin on DEET in rat plasma, DEET did not significantly affect permethrin kinetics. This is could be due to the low dose of permethrin used compared to DEET, and to a rapid hydrolytic cleavage of permethrin, presumably by esterases. This trend of rapid cleavage of permethrin has been reported *in vivo* and in *in vitro* incubation with human plasma (Abu-Qare and Abou-Donia, 2000a; Abu-Qare and Abou-Donia, 2001d). Moss (1996) also reported that DEET inhibited hydrolysis of some but not all of cholinesterase inhibitors in German cockroaches.

In this study, DEET and permethrin were applied in 70% ethanol, and at different application sites on the back of the neck of rats. This was to avoid possible interaction at the application site. The findings suggest there was no significant effect of each compound on absorption of the other. Our findings of *in vivo* absorption of Coadministered DEET and permethrin do not agree with previous *in vitro* studies in which DEET was administered concomitantly with another insecticide or drug. When both DEET and permethrin were coadministred to rodent and pig skin *in vitro*, DEET significantly decreased permethrin absorption (Baynes et al., 1997), and significantly enhanced persistence of the pyrethroide insecticide fenitrothion by rat skin *in vitro* (Moody et al, 1987). Furthermore, addition of DEET to the gel of the drug methotrexate resulted in two-fold increased permeation of methotrexate into muscle within 4 h after

dosing in rabbits (Lu et al., 1997). The gradual decrease in rate of absorption of both DEET and permethrin starting 24 h after application could be due to the saturation of the application site at that time, or to their binding to skin constituents.

In summary, co-administration of permethrin and DEET increased DEET concentration in the plasma, and enhance its persistence in the body as evident from AUC_{plasma} following combined application. This effect may due to a competition between permethrin and DEET on certain metabolic pathway, thus reducing the DEET rate of metabolism. In a recent study in our laboratory, permethrin inhibited DEET dissipation following *in vitro* incubation by human liver microsomes (Abu-Qare and Abou-Donia, 2001d). In a previous study by Hoy et al. (2000a), cutaneous administration of 15 mg/kg of permethrin with 5 mg/kg of pyridostigmine bromide increased permethrin concentration in serum of male and female rats compared to permethrin when applied alone at rate of 30 mg/kg. The findings could be correlated with previous and recent results from our laboratory that combined incubation of both compounds by human liver microsomes reduced rate of dissipation of DEET and permethrin compared to individual incubation, and might explain the observed enhanced neurotoxicity following concurrent application DEET and permethrin in hens (Abu-Qare and Abou-Donia, 2001d; Abou-Donia et al, 1996).

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Figure legends

Figure 1 HPLC profile of DEET, permethrin and their metabolites A) *m*-toulamide, B) *m*-toluic acid, C) DEET, D)*m*-phenoxybenzyl alcohol, E) *m*-phenoxybenzoic acid, and F) permethrin.

Figure 2 Suggested metabolic pathways of DEET following a single dermal dose in rats.

Figure 3 Proposed metabolic pathways of permethrin following a single dermal dose in rats.

Figure.4 HPLC profile of urinary excretion following combined dermal dose of DEET and permethrin: A) before hydrolysis B) After glucuronidase hydrolysis, C)After sulfatase hydrolysis.

Figure 5 Time course changes of DEET concentration in plasma of rats after a single dermal dose of 400 mg/kg.

Figure 6 Time course changes of permethrin concentration in plasma of rats after a single dermal dose of 1.3 mg/kg.

Figure 7 Time course changes of DEET concentration in plasma of rats after a single dermal dose of 400 mg/kg, and 1.3 mg/kg of permethrin.

Figure 8 Time course changes of permethrin concentration in plasma of rats after a single dermal dose of 1.3 mg/kg, and 400 mg/kg of DEET.

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Table.1 Retention[a] of DEET[b] and permethrin by rat skin following a single dermal dose of 400 mg/kg of DEET, and 1.3 mg/kg of permethrin, alone and in combination in Sprague-Dawley rats.

Time (h)	DEET	Permethrin	DEET in the presence of permethrin	Permethrin in the presence of DEET
0.5	45.6±7.8	62.3±13.4	51.3±9.0	72.6±8.5
1	32.7±12.6	57.8±10.9	43.8±9.8	65.6±6.7
2	28.6±6.5	49.2±7.6	36.5±5.6	54.6±7.2
4	22.1±8.2	38.9±12.6	30.1±8.7	45.6±7.2
8	18.7±9.6	29.7±6.3	25.6±9.0	33.7±5.9
24	11.2±5.6	21.1±10.9	18.6±5.6	18.9±7.0
48	1.5±1.2	11.7±5.4	5.3±4.5	9.1±4.6
72	0.05±0.03	4.2±1.5	0.5±0.3	6.1±3.0

[a] Results are expressed as percentage of the applied dose (mean±SD) of five animals.
[b] DEET and permethrin were applied in ethanol.

Table.2 Tissue concentration[a] of DEET and metabolites following a single dermal dose of 400 mg/kg in Sprague-Dawley rats.

	DEET					*m*-Toluic acid			*m*-Toluamide		
Time (h)	Plasma	Liver	Kidney	Brain	Testes	Plasma	Liver	Kidney	Plasma	Liver	Kidney
0.5	1472±361	921±219	891±721	241±101	N.D	241±65	202±131	98±73	132±96	169±102	34±26
1	1536±645	1204±532	956±207	247±123	N.D	313±148	119±75	112±76	245±93	301±163	101±64
2	1792±823	1650±302	1460±372	351±112	N.D	401±230	102±56	134±82	387±128	486±321	183±58
4	2103±631	1832±534	1609±237	260±134	N.D	391±90	187±69	174±101	460±153	512±201	290±259
8	1776±405	2209±709	1967±452	210±108	N.D	301±113	112±64	110±32	512±234	601±187	124±23
24	1294±320	1645±497	1830±643	198±93	181±54	109±76	N.D	125±68	460±254	245±123	N.D
48	852±209	1034±420	1273±297	76±34	165±63	N.D	N.D	N.D	N.D	N.D	N.D
72	276±132	354±98	675±245	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D

[a] Results are expressed as ng/g fresh tissue or ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).

[b] DEET was dissolved in ethanol.

Table.3 Tissue concentration[a] of permethrin[b] and metabolites following a single dermal dose of 1.3 mg/kg in Sprague-Dawley rats.

	Permethrin					*m*-Phenoxybenzyl alcohol			*m*-Phenoxybenzoic acid		
Time (h)	Plasma	Liver	Kidney	Brain	Testes	Plasma	Liver	Kidney	Plasma	Liver	Kidney
0.5	46±13	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
1	94±16	N.D	148±42	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
2	97±41	65±27	153±29	N.D	N.D	56±12	N.D	42±19	102±13	73±8.6	N.D
4	103±37	59±31	183±101	N.D	N.D	93±31	71±23	56±13	97±9.5	82±17	N.D
8	130±29	76±35	185±92	49±32	N.D	57±10	68±41	59±20	83±16	89±26	72±9.2
24	193±54	63±12	109±63	52±27	N.D	N.D	63±19	73±16	132±52	126±40	63±13
48	112±51	N.D	73±31	N.D	N.D	N.D	78±36	68±23	121±26	123±28	78±21
72	N.D	N.D	68±29	N.D	N.D	N.D	N.D	49±21	N.D	68±13	N.D

[a] Results are expressed as ng/g fresh tissue or ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).

[b] Permethrin was dissolved in ethanol.

Table.4 Tissue concentration[a] of DEET[b] and metabolites following concurrent application of a single dermal dose of 400 mg/kg of DEET, and 1.3 mg/kg of permethrin in Sprague-Dawley rats.

	DEET					*m*-Toluic acid			*m*-Toluamide		
Time (h)	Plasma	Liver	Kidney	Brain	Testes	Plasma	Liver	Kidney	Plasma	Liver	Kidney
0.5	1391±601	1126±349	931±218	301±97	N.D	183±76	213±138	113±29	189±18	156±53	91±63
1	1382±362	1163±521	1073±308	273±101	N.D	291±118	224±109	146±53	269±87	297±101	176±23
2	1613±247	1593±367	1317±418	296±114	N.D	392±78	163±78	172±47	382±156	397±162	218±49
4	2063±214	1901±461	1729±328	243±108	N.D	461±193	149±61	162±53	496±218	523±297	293±116
8	2416±608	2314±561	1923±216	212±93	93±12	482±213	109±45	116±35	559±296	591±213	217±63
24	2018±538	2102±391	1971±273	172±81	216±72	135±126	87±28	N.D	362±129	263±116	81±45
48	1046±291	967±218	1091±259	83±29	N.D	N.D	N.D	N.D	N.D	N.D	N.D
72	358±216	193±91	416±138	N.D	N.D	N.D	N.D	N>D	N.D	N.D	N.D

[a] Results are expressed as ng/g fresh tissue or ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).
[b] DEET and permethrin were dissolved in ethanol.

Table.5 Tissue concentration[a] of permethrin[b] and metabolites following concurrent application of a single dermal dose of 1.3 mg/kg of permethrin and 400 mg/kg of DEET in Sprague-Dawley rats.

	Permethrin					*m*-Phenoxybenzyl alcohol			*m*-Phenoxybenzoic acid		
Time (h)	Plasma	Liver	Kidney	Brain	Testes	Plasma	Liver	Kidney	Plasma	Liver	Kidney
0.5	48±14	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
1	87±29	N.D	N.D	N.D	N.D	N,.D	N.D	N.D	N.D	N.D	N.D
2	103±47	73±68	92±53	N.D	N.D	72±39	N.D	64±18	113±26	92±36	N.D
4	123±62	102±37	136±29	57±34	N.D	101±23	78±61	83±22	108±47	106±24	N.D
8	142±76	113±42	172±32	68±42	N.D	69±41	83±42	108±29	147±39	90±27	126±49
24	187±56	78±29	111±28	59±31	N.D	52±13	71±26	127±53	97±19	83±42	107±23
48	97±13	N.D	68±19	53±27	N.D	N.D	63±19	98±60	73±21	N.D	101±57
72	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D

[a] Results are expressed as ng/g fresh tissue of ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).
[b] Permethrin and DEET were applied in ethanol.

Table.6 Pharmacokinetic parameters[a] of DEET in rat plasma following a single dermal dose of 400 mg/kg, alone and in combination with 1.3 mg/kg of permethrin.

	AUC (ng.hr/ml)	C_{max} (ng/ml)	T_{max} (hr)	$T_{1/2}$ (hr)	K_{10} (hr^{-1})
DEET alone	96985±10763	2103±631	2.3±0.5	32.6±6.3	0.02±0.004
DEET, in the presence of permethrin	133345±11703	2416±261	4.9±1.3	36.1±12.8	0.019±0.007

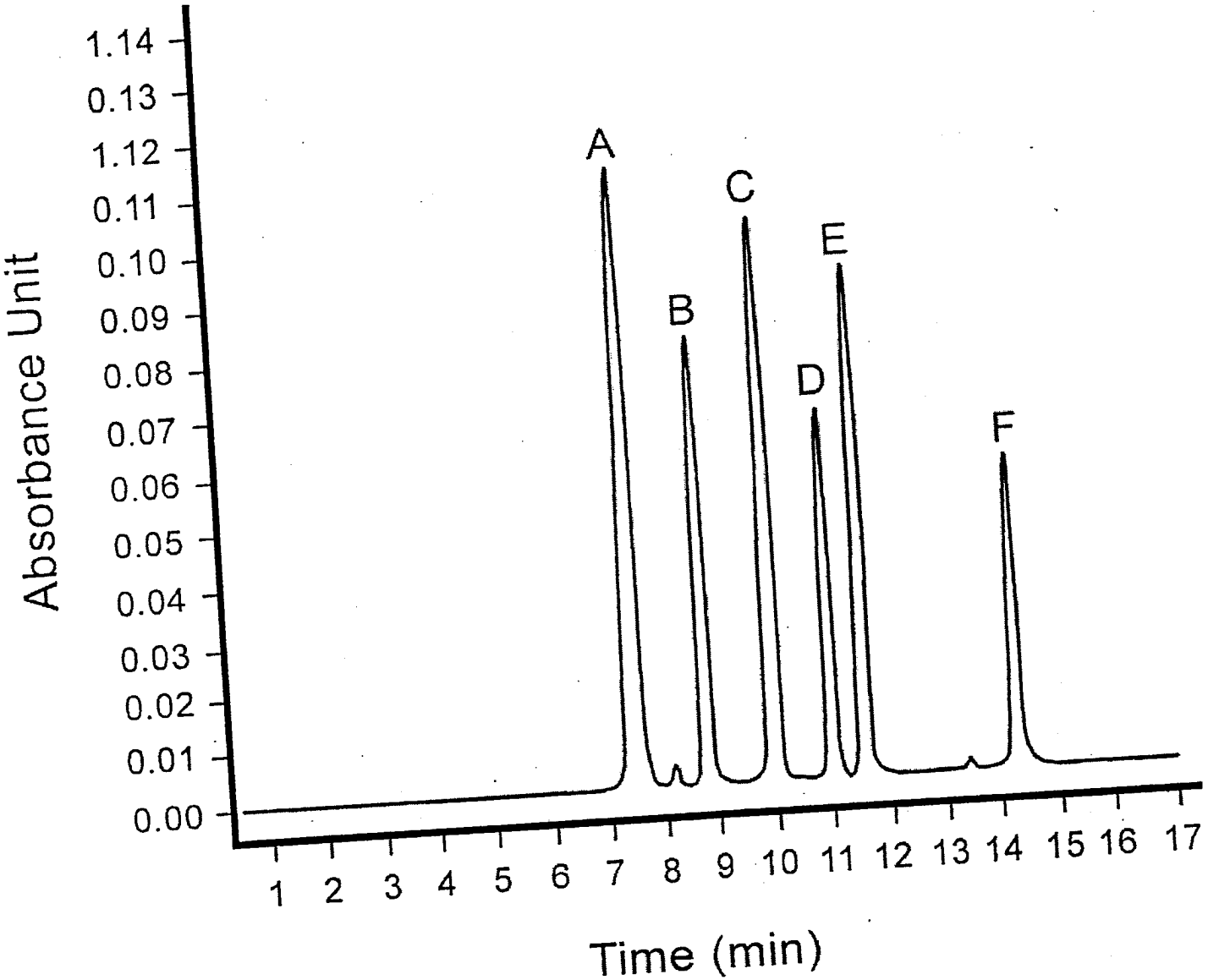
[a] All parameters are defined under methods.

Table.7 Pharmacokinetic parameters[a] of permethrin in rat plasma following a single dermal dose of 1.3 mg/kg, alone and in combination with 400 mg/kg of DEET.

	AUC (ng.hr/ml)	C_{max} (ng/ml)	T_{max} (hr)	$T_{1/2}$ (hr)	K_{10} (hr^{-1})
Permethrin alone	10502±2859	193.7±54	15.65±3.7	22.9±5.9	0.033±0.0026
Permethrin, in the presence of DEET	10637±2849	187.5±56	14.7±2.9	25.7±8.5	0.023±0.011

[a] All parameters are defined under methods.

Fig.1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0.13
1.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
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0.03
0.02
0.01
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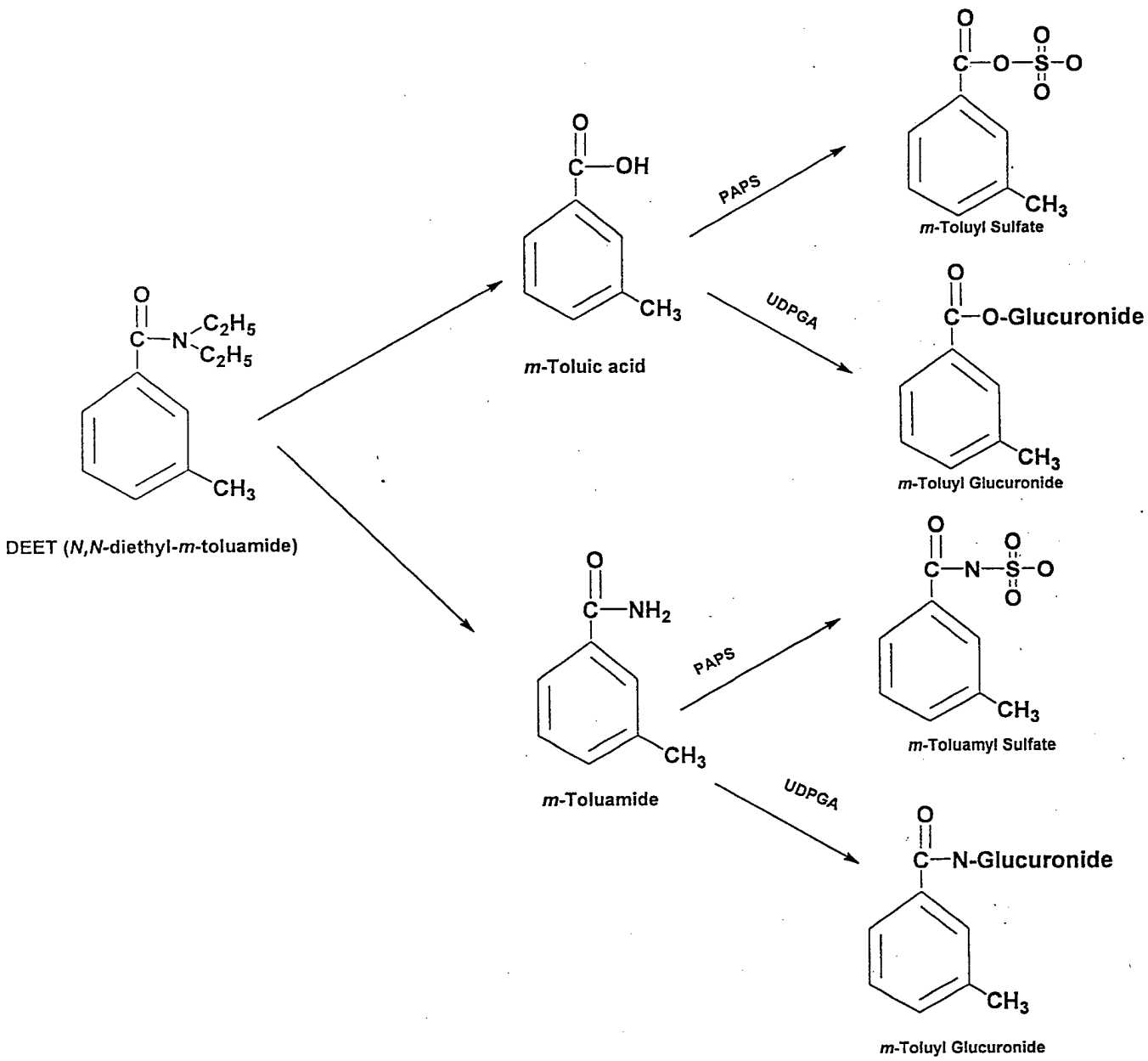
A
B
C
D
E
F
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Time (min)

Fig. 2


DEET (N,N-diethyl-m-toluamide)
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PAPS
m-Toluyl Sulfate
UDPGA
m-Toluyl Glucuronide
m-Toluamide
PAPS
m-Toluamyl Sulfate
UDPGA
m-Toluyl Glucuronide

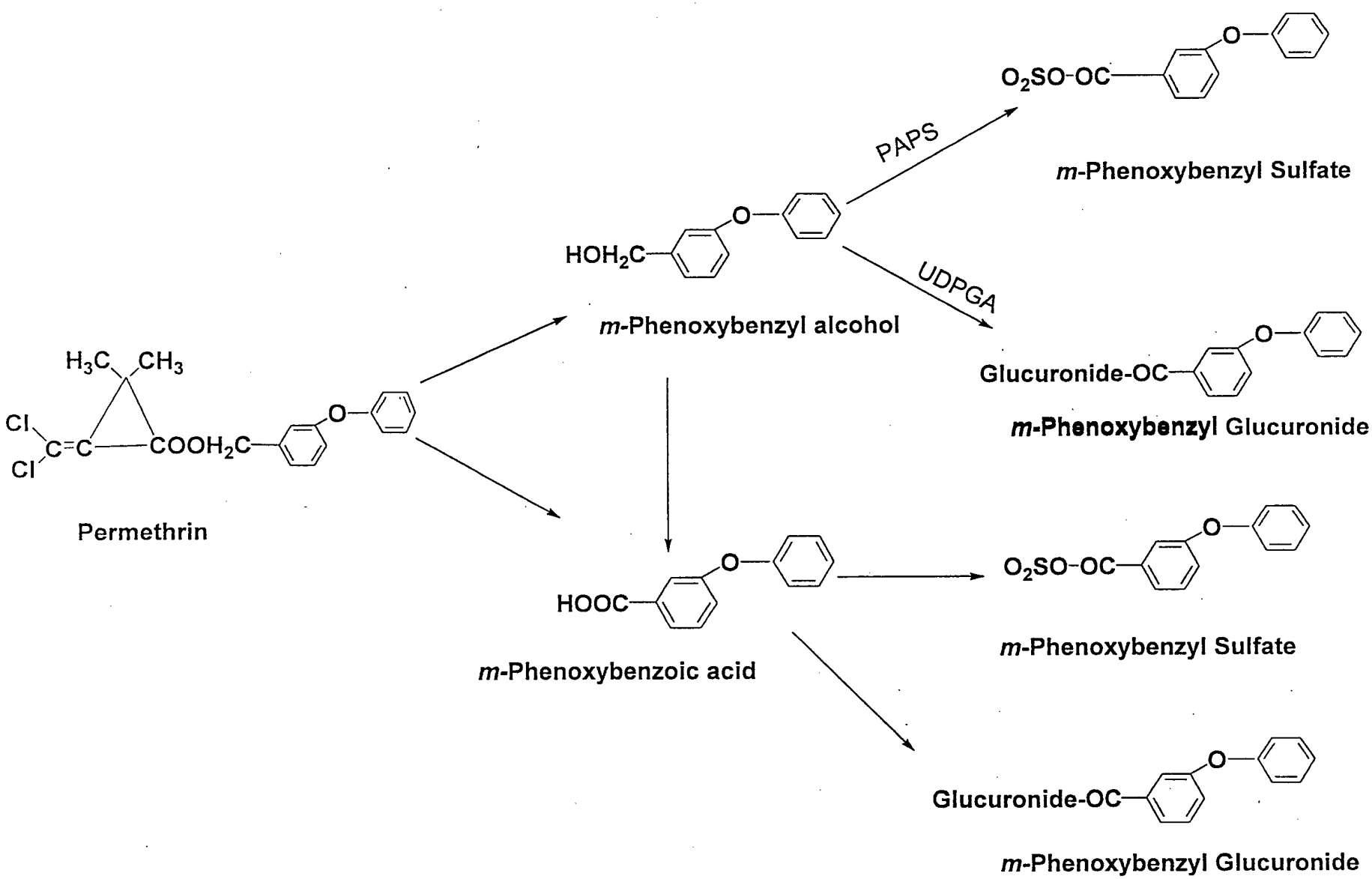
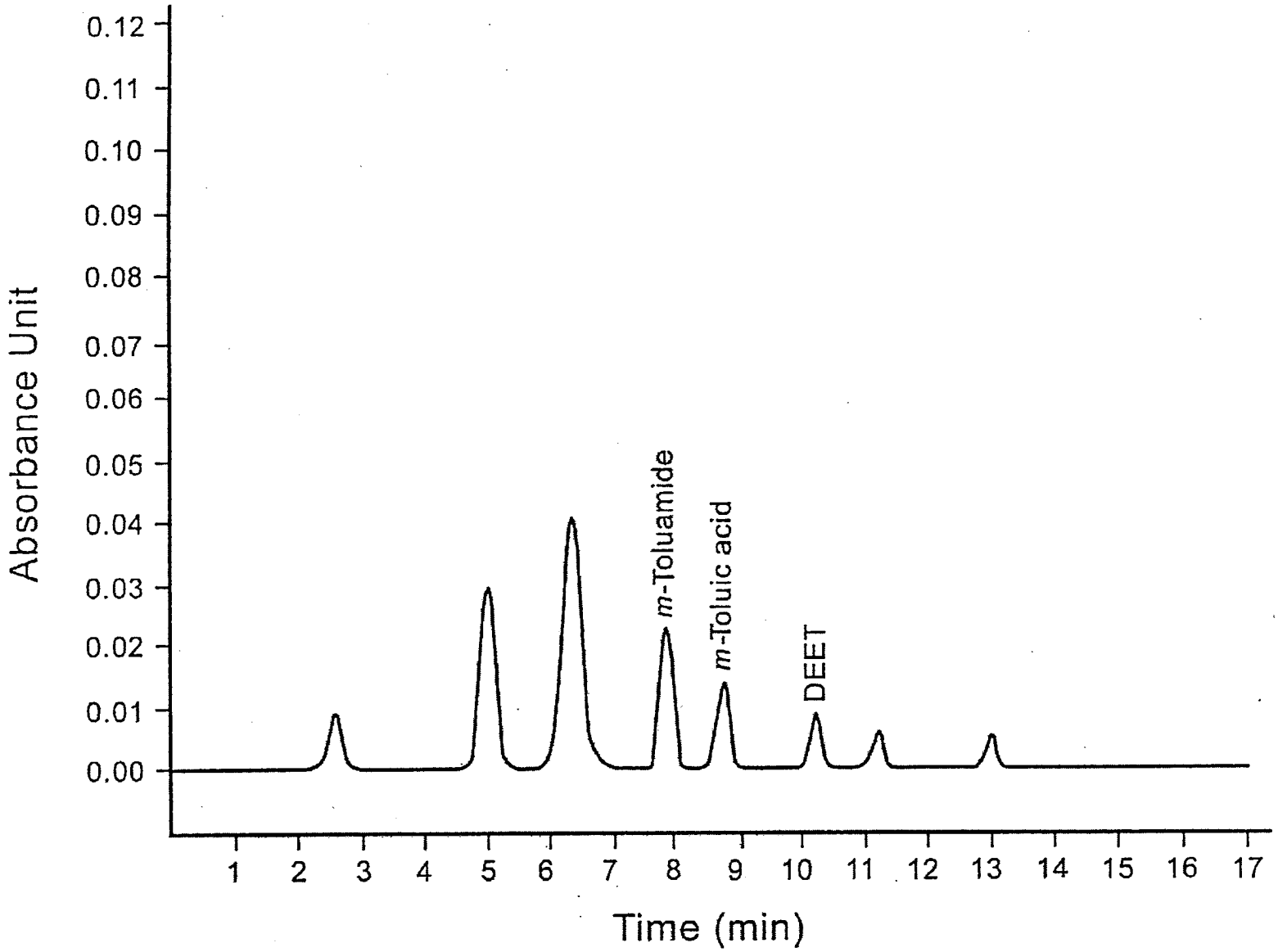

Permethrin
m-Phenoxybenzyl alcohol
PAPS
UDPGA
m-Phenoxybenzyl Sulfate
Glucuronide-OC
m-Phenoxybenzyl Glucuronide
m-Phenoxybenzoic acid
m-Phenoxybenzyl Sulfate
Glucuronide-OC
m-Phenoxybenzyl Glucuronide

Fig. 4A


Absorbance Unit
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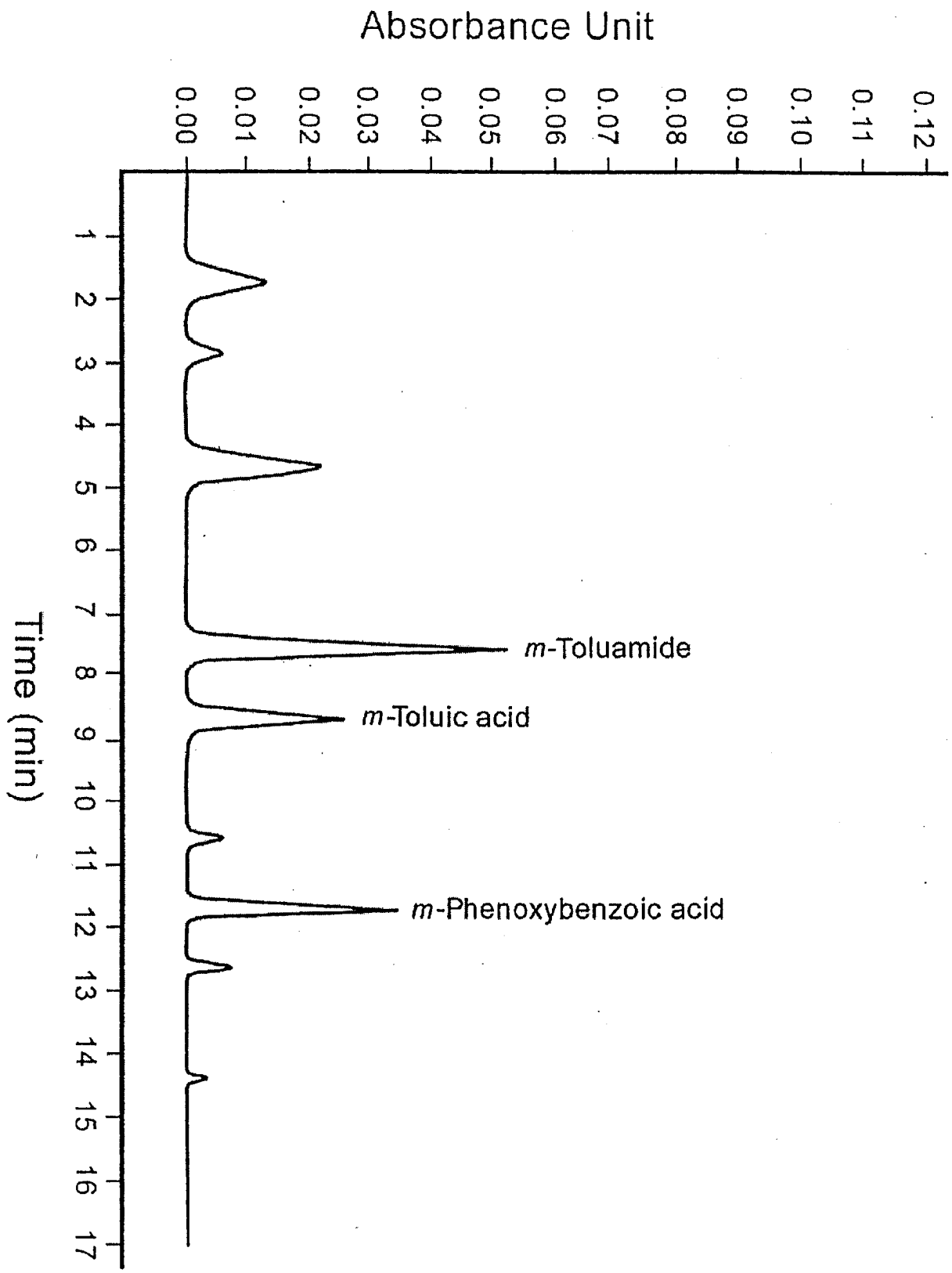
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
m-Toluamide
m-Toluic acid
DEET
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Time (min)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0.03
0.04
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0.06
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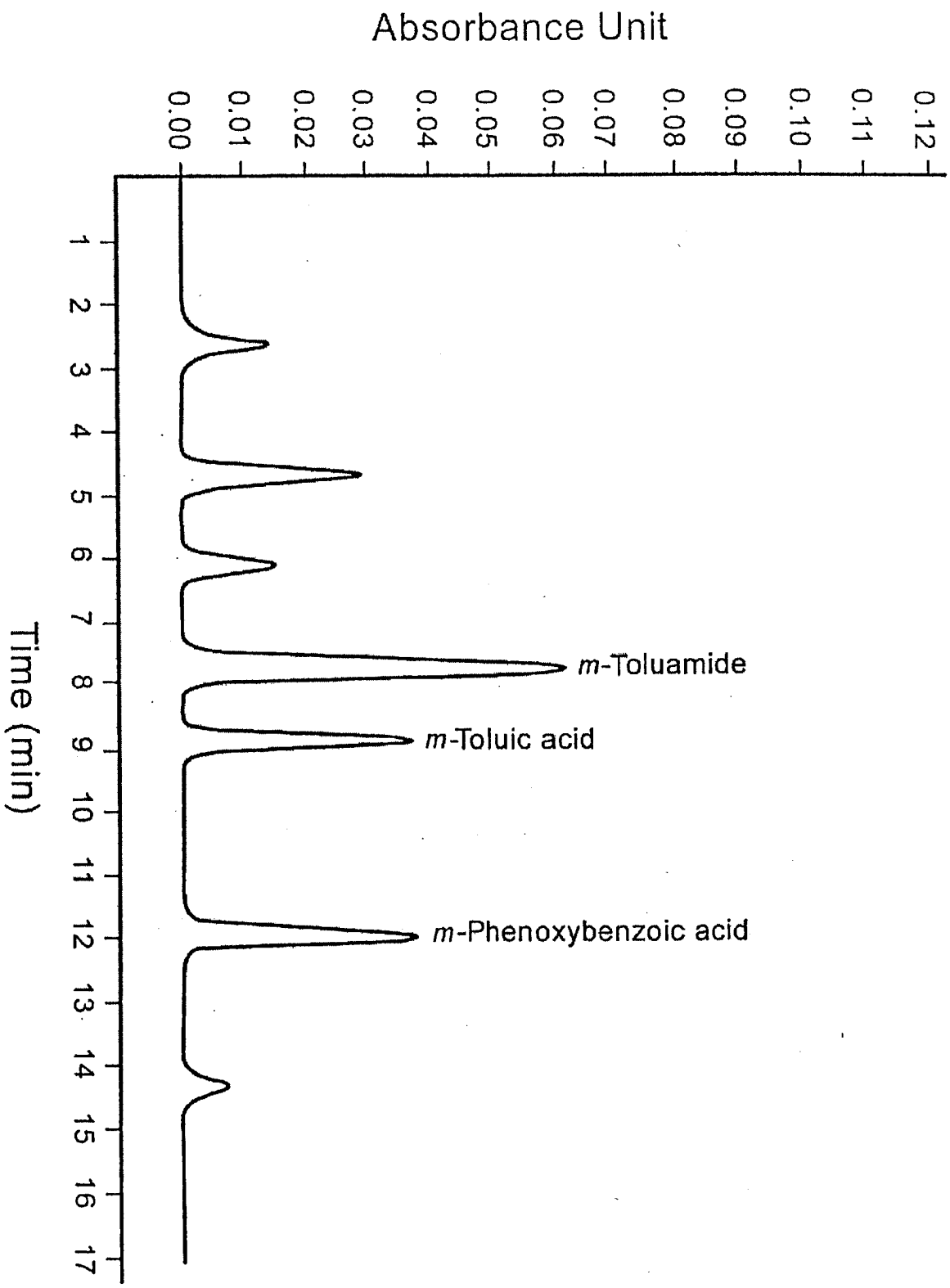
0.08
0.09
0.10
0.11
0.12
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
m-Toluamide
m-Toluic acid
m-Phenoxybenzoic acid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0.02
0.03
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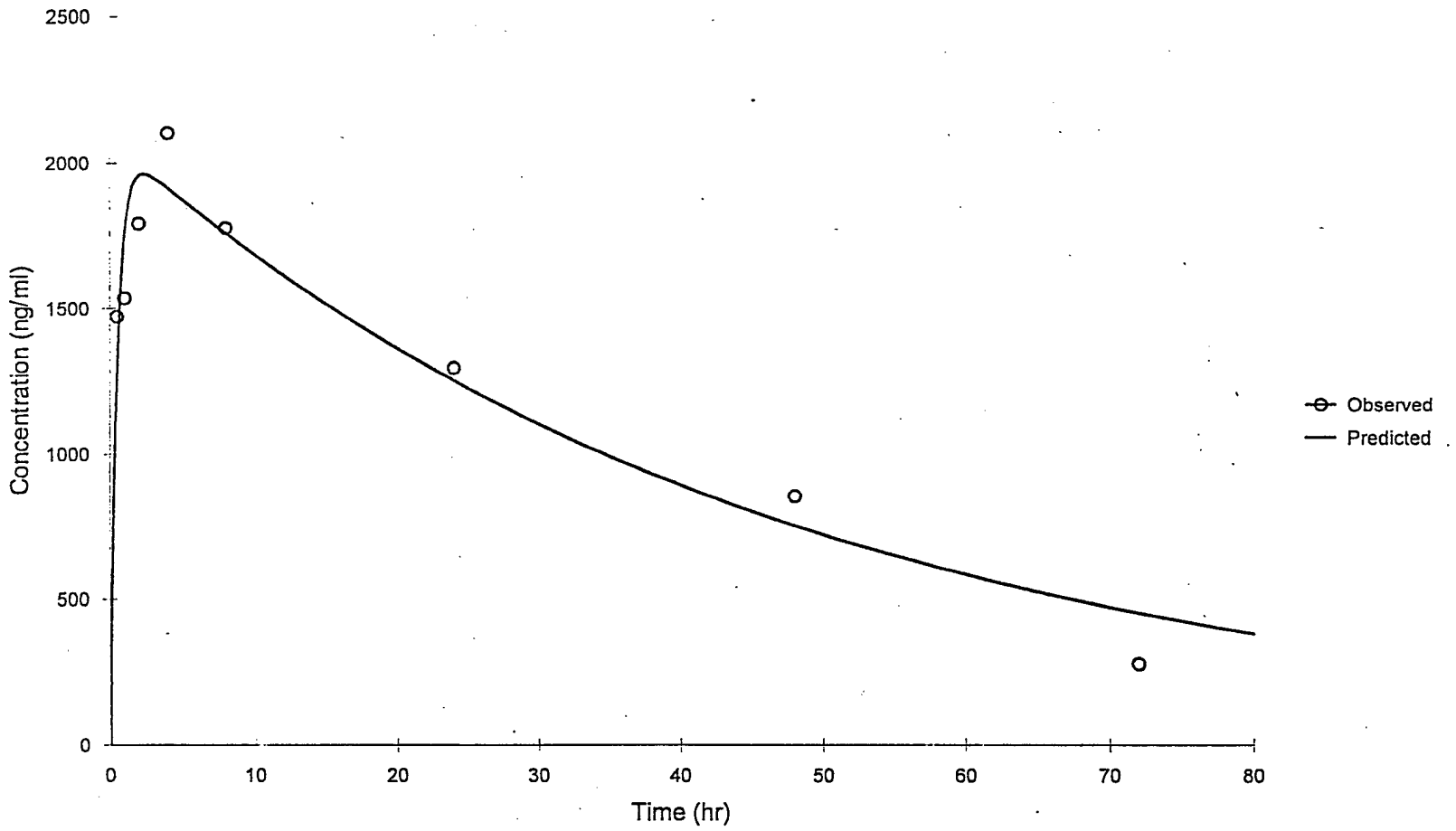
0.10
0.11
0.12
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
m-Toluamide
m-Toluic acid
m-Phenoxybenzoic acid


2500
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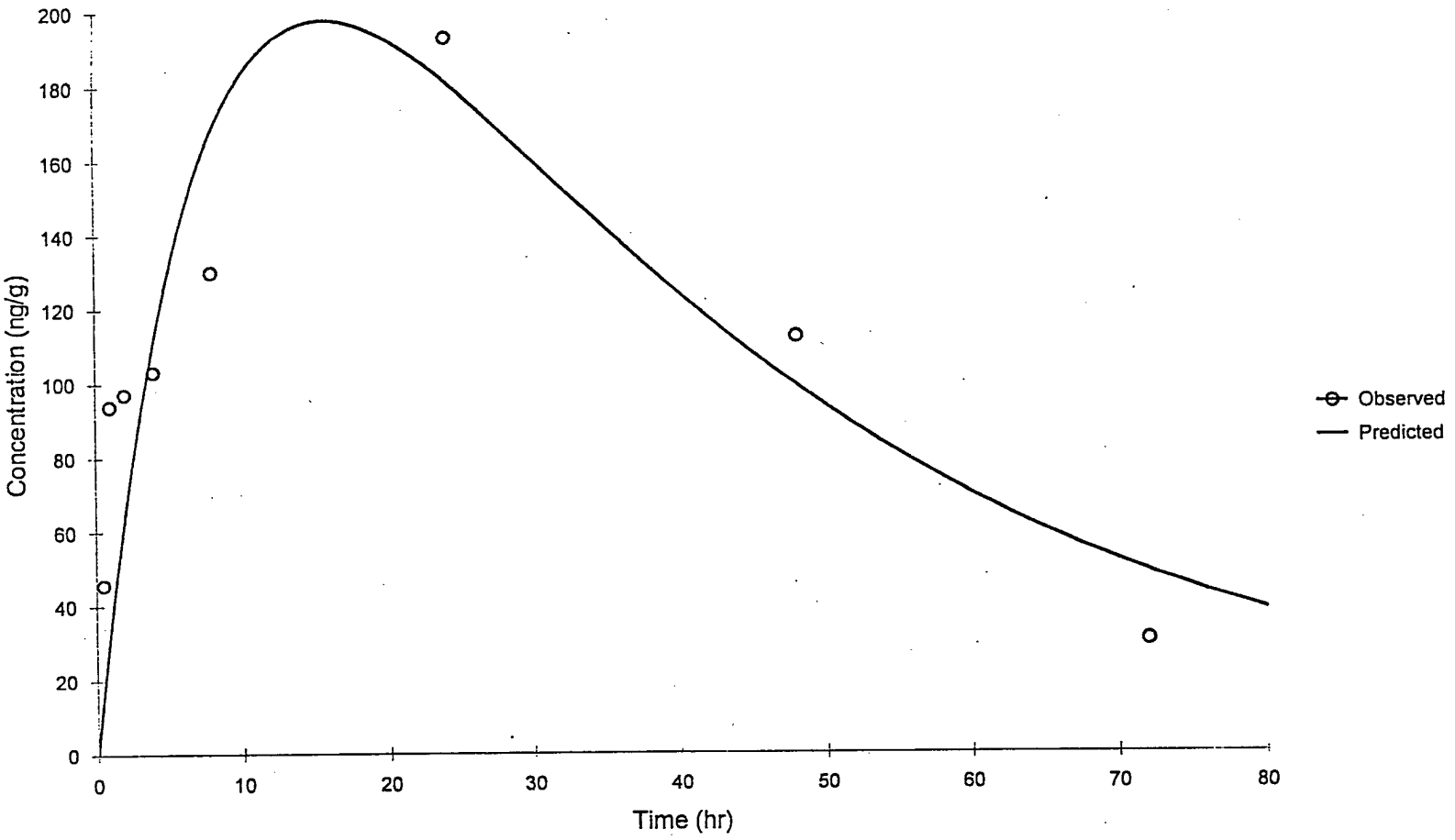
1500
1000
500
0
Concentration (ng/ml)
0
10
20
30
40
50
60
70
80
Time (hr)
Observed
Predicted


Concentration (ng/g)
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Time (hr)
Observed
Predicted

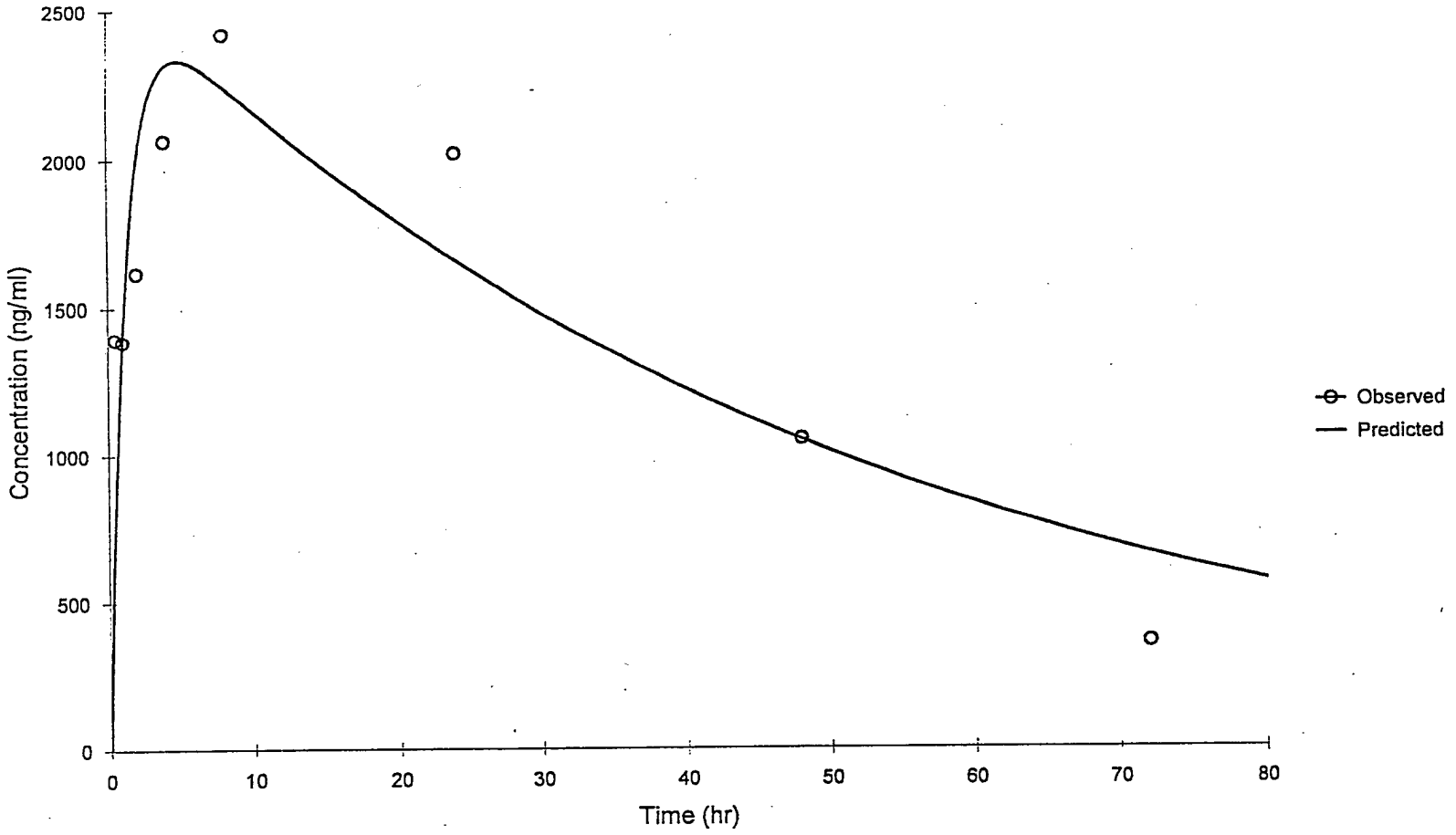
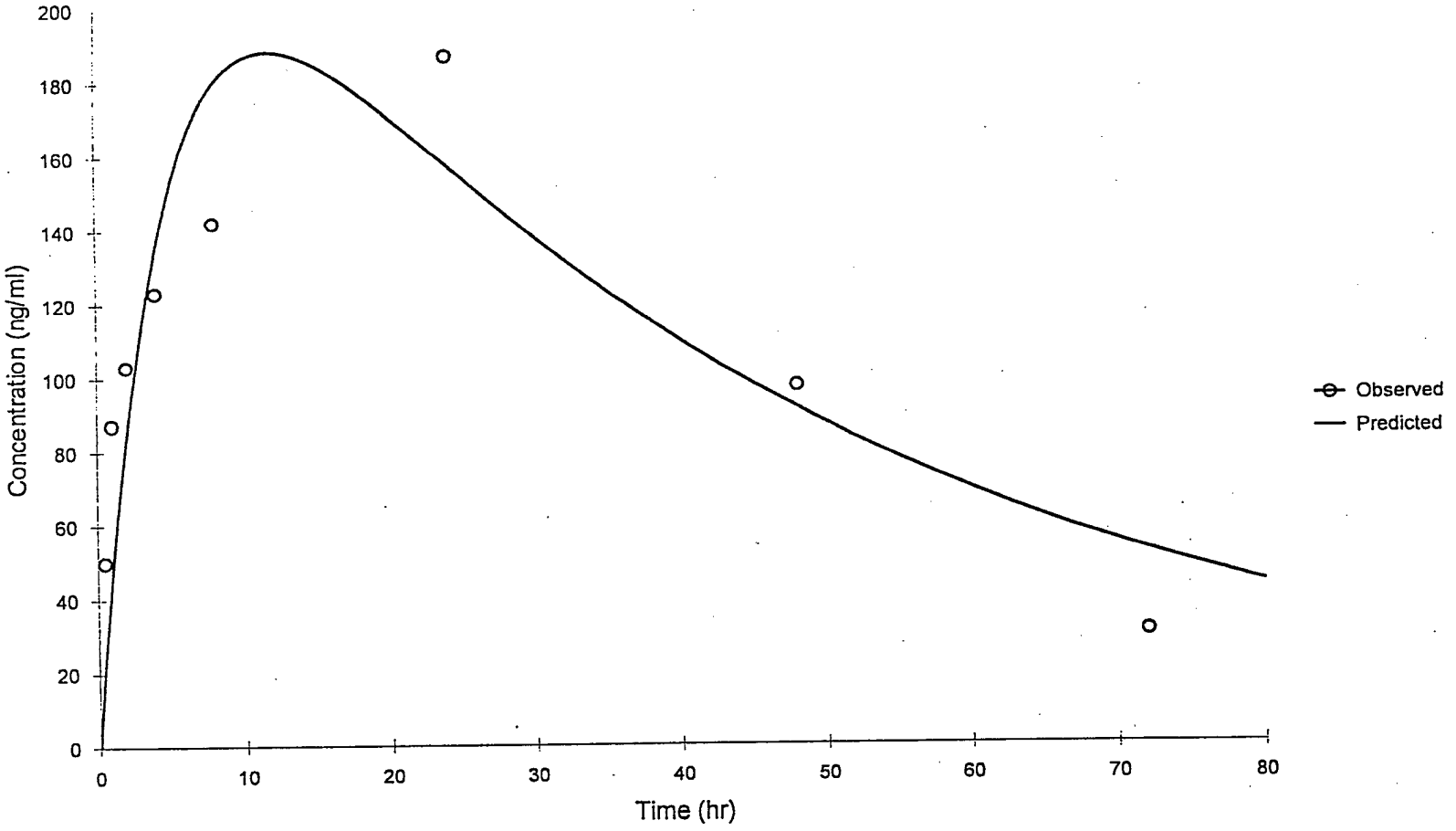

2500
2000
1500
1000
500
0
Concentration (ng/ml)
0
10
20
30
40
50
60
70
80
Time (hr)
Observed
Predicted

Fig. 8


Concentration (ng/ml)
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Time (hr)
Observed
Predicted

Appendix 4
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Mohamed B. Abou-Donia

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JOURNAL OF CHROMATOGRAPHY B

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Simultaneous determination of pyridostigmine bromide, *N,N*-diethyl-*m*-toluamide, permethrin, and their metabolites in rat plasma and urine by high-performance liquid chromatography

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Abstract

A rapid and simple method was developed for the separation and quantification of the anti nerve agent drug pyridostignmine bromide (PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide) its metabolite *N*-methyl-3-hydroxypyridinium bromide, the insect repellent DEET (*N,N*-diethyl-*m*-toluamide), its metabolites *m*-toluamide and *m*-toluic acid, the insecticide permethrin (3-(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylic acid(3-phenoxyphenyl)methy-lester), and two of its metabolites *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid in rat plasma and urine. The method is based on using C_{18} Sep-Pak® cartridges for solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) with reversed-phase C_{18} column, and gradient UV detection ranging between 208 and 230 nm. The compounds were separated using gradient of 1 to 99% acetonitrile in water (pH 3.20) at a flow-rate ranging between 0.5 and 1.7 ml/min in a period of 17 min. The retention times ranged from 5.7 to 14.5 min. The limits of detection were ranged between 20 and 100 ng/ml, while limits of quantitation were 150–200 ng/ml. Average percentage recovery of five spiked plasma samples were 51.4±10.6, 71.1±11.0, 82.3±6.7, 60.4±11.8, 63.6±10.1, 69.3±8.5, 68.3±12.0, 82.6±8.1, and from urine 55.9±9.8, 60.3±7.4, 77.9±9.1, 61.7±13.5, 68.6±8.9, 62.0±9.5, 72.9±9.1, and 72.1±8.0, for pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, respectively. The relationship between peak areas and concentration was linear over the range between 100 and 5000 ng/ml. This method was applied to analyze the above chemicals and metabolites following their administration in rats.

Keywords: Pyridostigmine bromide; *N,N*-Diethyl-*m*-toluamide; Permethrin

1. Introduction

Simultaneous exposure to pyridostigmine bromide, DEET and permethrin has resulted in enhanced of neurotoxicity in hens [1], and caused significant increase in lethality in rats [2]. Also acute interaction resulted in seizures and death following combined application of pyridostigmine bromide and DEET in mice [5]. Based on these reports, combined chemical exposure has been proposed as a possible cause of Gulf War veterans illness [1,3–8]. Pyridostigmine bromide was used as an antidotal drug against possible attack by organophosphate nerve agents, DEET was applied as insect repellent on the skin of

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PII: S0378-4347(00)00407-2

veterans, and permethrin was sprayed against orthropod vectors on the battle dress uniforms [35]. Pyridostigmine bromide has been reported to be absorbed into plasma and excreted in urine following oral or intravenous dose in rat [9,10,17,18,27,29,36], in man [16,24], and in dog [15,28]. Absorption and excretion of DEET and metabolites were rapid after dermal application in human [11,13], in rats [19], and in dogs [14]. Permethrin was also reported to be absorbed into plasma, metabolized and excreted as metabolites in the urine following oral or intravenous dose in rats [20], and in rabbits [23].

Several analytical methods have been used for identification and quantification of the above chemicals and their metabolites, when applied alone in plasma and urine samples. These methods used high-performance liquid chromatography (HPLC) [9,11–14,25,34], HPLC–mass spectrometry [19], gas chromatography [21–24,39–41], gas chromatography–mass spectrometry [26,32,38,42], and thin layer chromatography [22,30,31]. Other techniques were also used, e.g. Micellar electrokinetic chromatography (MEKC) [33], radiochromatoelectrophoresis [34], electrophoresis with paper chromatography [36], and radioaminoassay [36]. Limits of detection of the chemicals and metabolites in plasma or urine samples when analyzed using HPLC–UV, following individual application were ranged between 10 and 100 ng/ml [11,20,25], while their recoveries were between 65 and 95% [20,25,37].

In this study we present a reliable method for simultaneous analysis of the above chemicals and their metabolites in rat plasma and urine using solid-phase extraction (SPE) coupled with reversed-phase HPLC (RP-HPLC).

2. Experimental

2.1. Chemicals and materials

DEET (*N,N*-Diethyl-*m*-toluamide) (Fig. 1) was obtained from Aldrich Chem Co., Inc. (Milwankee, WI, USA), Pyridostigmine bromide (PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide), *m*-phenoxybenzoic acid, and *m*-phenoxybenzyl alcohol (Fig. 1) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Permethrin(3-(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylicacid(3-phenoxyphenyl) methylester) was obtained from Chem Service, Inc. (West Chester, PA, USA), *m*-Toluamide, and *m*-toluic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA), *N*-methyl-3-hydoxypyridinium bromide was prepared following the method by Somani et al. [16]. Water (HPLC grade) and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Paris, Kentucky, USA). C_{18} Sep-Pak^{R} cartridges were obtained from Waters Corporation (Waters Corporation, Milford, MA, USA).

2.2. Animals

Rats (Sprague–Dawley) were purchased from Zivic Miller (Zelienople, PA, USA). The animals were kept in plastic metabolic cages. Three groups, each of five rats were treated with a single oral dose of 13 mg/kg of pyridostigmine bromide, a single dermal dose 400 mg/kg of DEET, and a single dermal dose of 1.3 mg/kg of permethrin. Another group of five rats were treated with a combination of the above chemicals. Five untreated control rats were treated with oral dose of water, or dermal dose of ethanol. The animals were held in metabolic cages allow collection of urine samples. Urine samples were collected from treated and control rats after 8 h of dosing. The animals were anesthetized with halothane and scarified by heart exsanguinations at 8 h. Blood was collected via heart puncture with a heparinized syringe and centrifuged at 2400 rev./min for 15 min at 5°C to separate plasma. Urine and plasma samples were stored at −20°C prior to analysis.

2.3. Instrumentation

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E Multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector, and a Waters 2487 Dual λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2 cm×4.0 mm, 5 μm (Supelco Park, Bellefonte, PA), and a reversed-phase C_{18} column μBondapak™ C_{18} 125A° 10 μm, 3.9×300 mm were used, (Waters Corporation, Milford, MA).

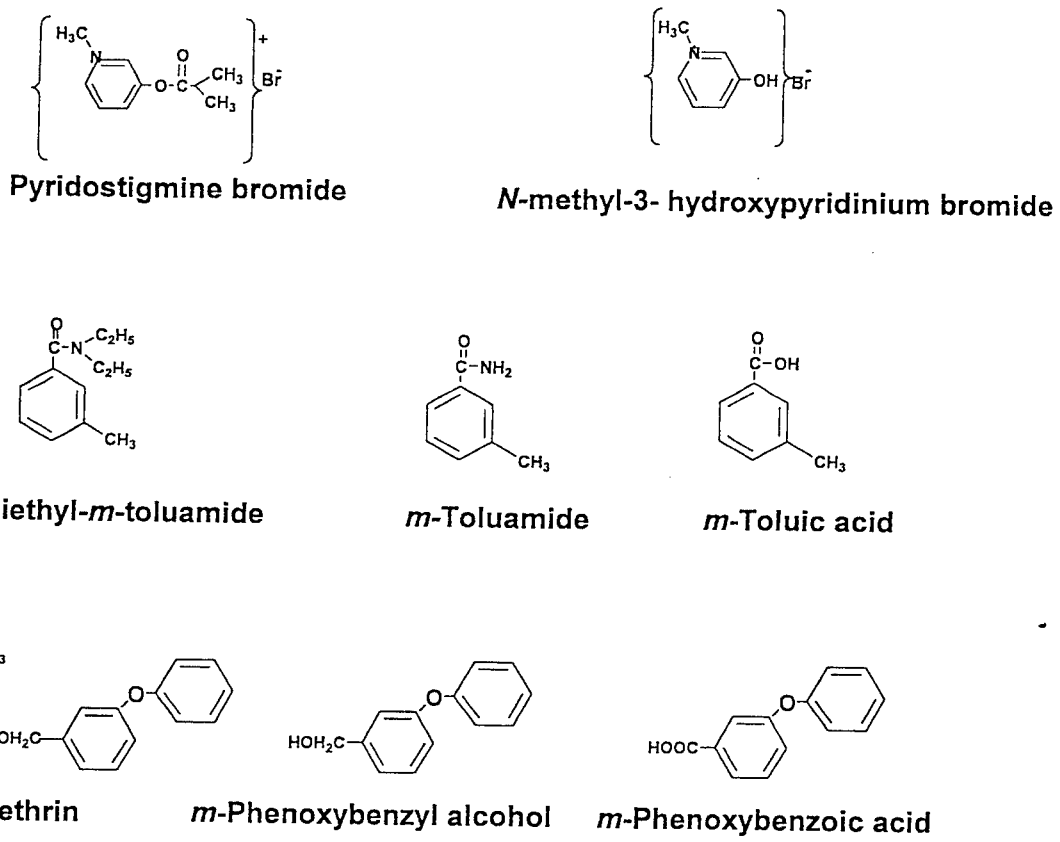

Fig. 1. Chemical structures of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid.

2.4. Sample preparation

A 0.2 ml plasma and urine samples from untreated rats were spiked with concentrations ranging between 100 and 5000 ng/ml of each of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzoic acid, and *m*-phenoxybenzyl alcohol. Spiked and treated samples were acidified with 1 N acetic acid (pH 5.0). Disposable C_{18} Sep-Pak Vac 3cc (500 mg) cartridges (Waters Corporation, Milford, MA) were conditioned with 3 ml of acetonitrile, then equilibrated using 3 ml of water prior to use. The spiked urine and plasma samples were vortexed for 30 s, centrifuged for 5 min at 1000 *g*, and the supernatant was loaded into the disposable cartridges, then washed with 2 ml of water, and eluted two times by 1 ml of methanol, then twice by 1 ml of acetonitrile, and reduced to 500 μl using a stream of nitrogen, prior to analysis by HPLC.

2.5. Chromatographic conditions

A 10 μl solution of plasma or urine residues was injected into HPLC. The mobile phase was water (adjusted to pH 3.20 using 1 N acetic acid):acetonitrile gradient at flow-rate programmed from 0.5 to 1.7 ml/min. The gradient started at 1% acetonitrile, increased to 75% acetonitrile at 6 min, then increased to 99% acetonitrile by 11 min. Then the system returned to 1% acetonitrile at 15 min where it was kept under this condition for 2 min to re-equilibrate. The eluents were monitored by UV detection of wavelength of 208 nm for pyridostigmine bromide and *N*-methyl-3-hydroxypyridinium bromide, 210 nm for DEET, *m*-toluamide, and *m*-

toluic acid and at 230 nm for permethrin, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid. The chromatographic analysis was performed at ambient temperature.

2.6. Calibration procedures

Five different calibration standards of a mixture of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were prepared in acetonitrile. Their concentrations ranged from 100 to 5000 ng/ml. Linear calibration curves were obtained by plotting the peak areas of the individual chemicals as a function of the concentration using GraphPad Prism program for windows (GraphPad Software, Inc., San Diego, CA, USA). The standard curves were used to determine recovery of the chemicals from plasma and urine samples.

2.7. Limits of detection and limits of quantitation

Limits of detection (LOD) were determined at the lowest concentration to be detected, taking into consideration a 1:3 baseline noise: calibration point ratio. A reproducible lowest possible concentration was considered as the limit of quantitation (LOQ). The LOQ was repeated five times for confirmation.

3. Results

3.1. Standard calibration curves

The standard calibration curves of peak area against concentration of pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid are shown in Fig. 2. Linearity of the calibration curves for the three compounds was achieved at concentrations ranging from 100 to 5000 ng/ml.

3.2. Chromatogram

Chromatographic profiles were obtained for rat plasma and urine samples after solid-phase extraction using C_{18} Sep Pak® cartridges under HPLC conditions, described above (Figs. 3 and 4). Retention times were 6.8, 9.5, 14.4, 5.7, 7.5, 8.6, 10.7 and 11.3 min for pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively. The total run time was 17 min. Clean chromatogram shows no interference from endogenous substances in plasma and urine samples. This suggests an efficient sample preparation and clean up method.

3.3. Extraction efficiency and recovery

The average extraction recoveries of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzoic acid, and *m*-phenoxybenzyl alcohol were determined at concentrations ranged between 100 and 5000 μg/ml (Tables 1 and 2).

Spiked plasma and urine samples were extracted and analyzed for each concentration in five replicates. Average percentage recoveries were 51.4±10.6, 71.1±11.2, 82.3.±6.7, 60.4±11.8, 63.6±10.1, 69.3±8.5, 68.3±12.0 and 82.6±8.1 from plasma, and 55.9±9.8, 60.3±7.4, 77.9±9.1, 53.9±9.7, 64.2±6.5, 71.7±4.2, 86.5±6.1 and 89.7±4.1 from urine for pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively.

3.4. LOD

Blank plasma and urine samples from untreated rats were used as references for plasma and urine collections. LOD were calculated from a peak signal-to-noise ratio of 3:1. The resulting detection limits range were 100, 50, 50, 100, 100, 80, 20 and 30 for pyridostigmine, DEET, permethrin, *N*-methyl-3-hydroxypyridine, *m*-toluamide, *m*-toluic acid, *m*-phenoxy benzylalcohol, and *m*-phenoxy benzoic acid, respectively.

3.5. LOQ

LOQ were determined to be 150 ng/ml for pyridostigmine bromide and DEET and 100 ng/ml

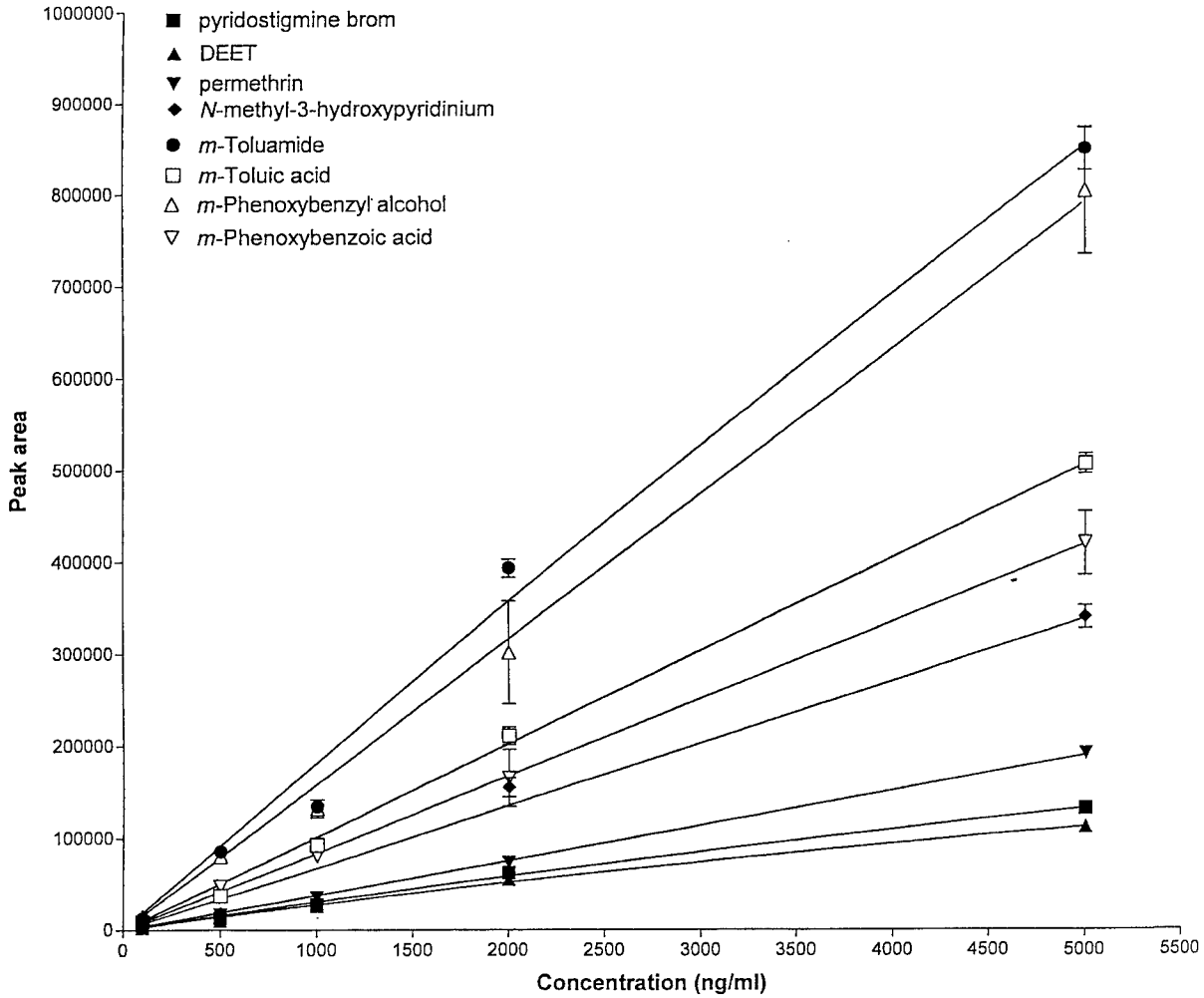

Fig. 2. Standard calibration curves of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid.

for permethrin in plasma. In urine limits of quantitation were 200, 150, and 100, 150, 150, 100, 100, 100 ng/ml for pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridine, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively.

3.6. Application of the method to biological samples

In order to validate the method, the method was applied for analysis of the chemicals in treated rats, when applied alone or in combination. The rats were sacrified at 8 h following dosing. In plasma, their levels were 224±123, 1320±346 and 182±76 ng/ml for pyridostigmine bromide, DEET, and permethrin, while concentration of metabolites in plasma were. 107.3±21.5, 98.4±14.8, 107.5±5.7 and 142.7±27.1 ng/ml for *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively. *N*-methyl-3-hydroxypyridinium bromide a metabolite of pyridostigmine bromide was not detected in rat plasma. Levels of pyridostigmine bromide and DEET in rat urine were 712±186 ng/ml and 3.2±0.82 μg/ml, respectively. DEET metabolites *m*-toluamide and *m*-toluic acid, and

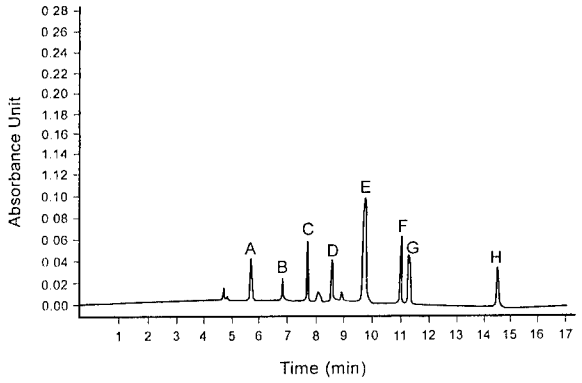

Fig. 3. Chromatogram of plasma sample of (A) *N*-methyl-3-hydoxypyridinium bromide; (B) PB (pyridostigmine bromide); (C) *m*-toluamide; (D) *m*-toluic acid; (E) DEET; (F) *m*-phenoxybenzyl alcohol; (G) *m*-phenoxybenzoic acid; and (H) permethrin under established HPLC conditions.

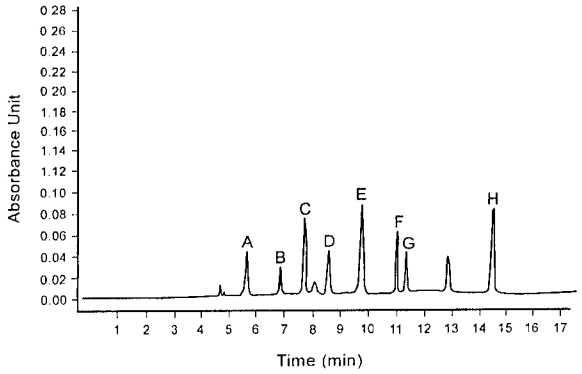

Fig. 4. Chromatogram of spiked urine sample with (A) *N*-methyl-3-hydoxypyridinium bromide; (B) PB (pyridostigmine bromide); (C) *m*-toluamide; (D) *m*-toluic acid; (E) DEET; (F) *m*-phenoxybenzyl alcohol; (G) *m*-phenoxybenzoic acid; and (H) permethrin under established HPLC conditions.

permethrin and its metabolites *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, have not been detected in urine after 8 h of dosing.

4. Discussion

The present study reports the development of an HPLC method for quantitative and qualitative analysis of pyridostigmine bromide, DEET, permethrin and their metabolites in plasma and urine of treated rats.

Linearity of standard calibration curves for the chemicals in the present method is in consistent with previous reports. Eilln et al. [9] reported linear range between 40 and 500 ng/ml for DEET in plasma using HPLC, while Yaylor et al. [14] reported a linearity over a range between 19 and 1910 ng/ml for DEET using gas chromatography (GC). Also, Chan et al. [38] reported a linear range for pyridostigmine bromide in human plasma over concentrations between 50 and 1000 ng/ml.

The chromatogram obtained following SPE and HPLC analysis shows no interference from plasma and urine subjects, indicating an efficient clean up method used. Also simultaneous and rapid analyses of the parent compounds and metabolites are cost efficient and save time for sample preparation.

Recoveries of the chemicals and metabolites were suitable for application of the method for analysis of treated samples for parent compounds and their metabolites. Low recovery for pyridostigmine bromide might have resulted from the use of solvent system that was not quite suitable for extracting pyridostigmine bromide, and at the same time it was needed for extracting and analyzing the other two

Table 1
Percentage recovery of pyridostigmine bromide, DEET permethrin, and metabolites in rat plasma[a]

Concentration (ng/ml)	Pyridostigmine bromide	DEET	Permethrin	*N*-methyl-3-hydroxypyriodinium bromide	*m*-Toluic acid	*m*-Toluamide	*m*-Phenoxybenzyl alcohol	*m*-Phenoxybenzoic acid
5000	61.8±5.4	81.4±4.3	81.4±7.6	53.4±13.6	61.5±12.1	73.4±13.6	65.7±18.2	89.2±9.7
2000	63.8±7.1	84.1±9.5	87.1±9.3	64.7±18.3	65.3±7.2	64.7±9.8	61.5±13.8	80.1±10.2
1000	55.5±10.7	73.6±8.7	82.1±8.3	57.1±9.2	68.4±17.6	70.2±4.1	68.7±10.2	73.6±8.3
200	58.4±13.6	71.2±4.8	72.1±6.7	59.8±7.4	60.5±13.8	71.8±8.6	75.3±8.2	86.7±6.4
100	44.1±10.9	60.7±16.7	71.5±8.9	66.2±10.4	62.1±4.6	66.2±6.5	70.4±9.7	83.4±5.8

[a] Values are expressed as mean±SD of five replicates.

Table 2
Percentage recovery of pyridostigmine bromide, DEET permethrin, and metabolites from rat urine[a]

Concentration (ng/ml)	Pyridostigmine bromide	DEET	Permethrin	*N*-methyl-3-hydroxypyridinium bromide	*m*-Toluamide	*m*-Toluic acid	*m*-Phenoxybenzyl alcohol	*m*-Phenoxybenzoic acid
5000	54.1±7.3	62.8±10.9	90.7±3.9	62.2±12.8	69.2±10.6	59.8±12.3	66.8±6.5	75.6±4.1
2000	53.8±8.4	58.1±7.5	83.1±4.2	52.7±10.6	61.9±9.7	63.4±8.7	73.8±12.3	71.9±12.1
1000	49.6±8.3	52.7±7.4	70.8±8.7	61.8±9.3	75.3±7.6	69.7±5.2	82.6±7.8	77.8±3.6
200	48.0±13.2	60.9±8.2	71.7±10.1	64.7±8.4	71.9±5.6	61.2±14.7	71.9±5.8	65.4±9.5
100	52.3±8.5	57.6±10.7	68.4±4.0	67.2±13.2	64.5±11.2	58.4±6.5	69.8±13.1	69.8±10.9

[a] Values are expressed as mean±SD of five replicates.

chemicals and metabolites under similar conditions. Also hydrolysis of pyridostigmine bromide during the extraction is possible, in a previous study Aquilonius and Hartvig [43] reported that extraction and analysis of pyrdostigmine bromide was a challenge to the analytical chemists, because of its in vitro hydrolysis could take place in buffer solutions, plasma and blood. Percentage recoveries depends on the matrix, extracting solvent, method of analysis, and the amount to be analyzed. Recoveries of DEET from serum and urine were reported to be 93–95%, and 65–70%, respectively using GC–MS as an analytical technique [38], while recovery of DEET from water samples was 45.6% using Micllar kinetic chromatography method [33]. Hennis et al. [15] reported a recovery of 50% of *N*-methyl-3-hydroxypyridinium from dog plasma and urine, while Chan et al. [37] reported a recovery of 82% of pyridostigmine bromide from plasma at low concentration of 50 ng/ml, while its recovery was 92% when a concentration of 400 ng/ml was used. In previous studies, recovery of pyrethroides and metabolites from rat urine ranged between 90 and 98% using GC–MS [26], while the recovery was 92% at high concentration of 400 ng/ml using GC. In our method, recoveries differed with individual chemicals. Recoveries of the chemicals analyzed in our method was between 55 and 83%. This range lies within the reported values in the literature, taking into consideration simultaneous analysis of the parent chemicals and their metabolites.

The LOD reported in our method allow to analyzing samples from treated animals following doses resemble real exposure. Our ability to detect the three compounds and metabolites in plasma after 8 h of dosing is an evidence of the method suitability. LOD and LOQ depended upon the nature of the matrix, rate of application, and method of analysis [10,38–40]. Pyridostigmine bromide and DEET were also detected in urine samples at 8 h, while failure to detect permethrin and metabolites in urine might be due to the low dermal dose of permethrin that used (1.3 mg/kg), its low absorption through skin, and to rapid hydrolysis and conjugation of permethrin and the targeted metabolites. Hennis et al. [15] reported a 50 ng/ml as a limit of detection of *N*-methyl-3-hydroxypyridinium in dog plasma using ion-exchange liquid chromatography, while Miller and Verma [36] reported a 2.5 ng/ml as detection limit of pyridostigmine bromide in tissues using radioimmunoassay method, while using HPLC technique, limits of detection of pyridostigmine bromide in plasma was 10 ng/ml [25], and ranged between 2.7 and 18.6 ng/ml in plasma using GC [41]. The detection limit of DEET was 90 and 90 ng/g from urine and serum, respectively, using HPLC–UV method [11], and 15 ng/ml for DEET in human and dog plasma using HPLC [34], while it was 25 ng in cosmetic products using high-performance thin-layer chromatography (HPTLC) method [30]. Detection limits of permethrin in urine samples were 0.3–0.5 μg/l using GC–MS technique [26], and 5 μg/l in plasma using GC method [39]. The reported LOD in the literature are consistent with our results for the simultaneous analysis of the combined chemicals and their metabolites, which ranged between 20 and 100 ng/ml.

A rapid and simple HPLC method was developed for separation and residual determination of pyridostigmine bromide, DEET, permethrin and selected metabolites in rat spiked and treated plasma and urine samples. SPE was used which selectively

extracted the above chemicals from plasma and urine samples without interference of an expected mixture of metabolites and endogenous compounds. The method could be applied routinely for monitoring of the above chemicals in human plasma and urine samples of persons exposed to the combined chemicals. This method could also be used in pharmacokinetics studies to assess distribution of the parent compounds and metabolites in body tissues and fluids. The use of SPE is advantageous compared to liquid–liquid extraction which is a time consuming and requires large amounts of organic solvents. The main advantage of the method is the ability to analyze simultaneously the three chemicals and their metabolites under similar conditions, saving time and expenses for sample preparation.

Acknowledgement

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Appendix 5
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Increased 8-hydroxy-2′-deoxyguanosine, a biomarker of oxidative DNA damage in rat urine following a single dermal dose of DEET (*N*,*N*-diethyl-*m*-toluamide), and permethrin, alone and in combination

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Abstract

Levels of the biomarker of DNA oxidative damage 8-hydroxy-2′-deoxyguanosine (8-OHdG) in rat urine following dermal exposure to DEET (*N*,*N*-diethyl-*m*-toluamide) and permethrin, alone and in combination have been determined. A group of five rats for each time point were treated with a single dermal dose of 400 mg/kg of DEET, 1.3 mg/kg of permethrin or their combination. Urine samples were collected 2, 4, 8, 16, 24, 48, and 72 h following application. Control urine samples of rats treated with ethanol were also collected at the same time intervals. Solid phase extraction coupled with high performance liquid chromatography (HPLC) with UV detection at 254 nm was used for determination of 2′-deoxyguanosine, and (8-OHdG). The limits of detection (LOD) were 0.5 ng of both 2′-deoxyguanosine and 8-OHdG. Their average percentage recoveries from urine samples were between 70–85%. A single dermal dose of DEET or in combination with permethrin significantly induced levels of (8-OHdG) that are excreted in the urine over the time course of the study compared to control urine samples. Permethrin did not cause significant increase in the amount of 8-OHdG in the urine. Levels of 8-OHdG in urine excreted at 24 h were 1009 ± 342, 1701 ± 321, 1140 ± 316, and 1897 ± 231 ng following treatment with ethanol, DEET, permethrin, and DEET + permethrin, respectively. The results indicate that dermal administration of DEET could generate free radical species hence cause DNA oxidative damage in rats.

Keywords: *N*,*N*-diethyl-*m*-toluamide; Permethrin; DNA oxidative damage; Free radicals

1. Introduction

Oxidative DNA damage is emerging as a biomarker of effect in studies assessing the health risks of occupational chemicals. DNA damage can lead to mutation, which can reflected in more severe biological consequences such as genetic

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disease and cancer (Josephy et al., 1997). Exogenous and endogenous oxidants frequently cause oxidative damage to DNA (Malins, 1993; Halliwell, 1999). Because the reactive oxidants are not suitable for analysis, oxidized bases like 8-hydroxy-2′-deoxyguanosine (8-OHdG) are used as biomarkers for DNA oxidative damage (Halliwell and Dizdaroglu, 1992; Loft et al. 1993; Kasai, 1997; Helbock et al., 1999; Anson et al., 2000). Determination level of (8-OHdG) in urine and tissues following exposure to environmental toxicants has been reported, e.g. *p*-dichlorobenzene (Umemura et al., 2000), trichloroethylene and perchloroethylene (Toraason et al., 1999), safrole (Liu et al., 1999), 2-nitropropane (Loft et al., 1998a) in rats, δ-aminolevulinic acid in Chinese hamster ovary (CHO) cells (Yusof et al., 1999), and dieldrin in fish (Rodriguez-Ariza et al., 1999). Also 8-OHdG was produced as a result of high fat diet in rats (Loft et al., 1998b), in tobacco smokers. (Howard et al., 1998), after exposure to copper in rats (Toyokuni and Sagripanti, 1994), and following exposure to *m*-phenylenediamine and its derivative in the presence of copper in humans (Chen et al., 1998), after acute iron intoxication in rat sperm cells in vivo and in vitro (Wellejus et al., 2000), and following incubation of Chinese hamster cells with metabolites of *o*-phenylphenol (Henschke et al., 2000). Furthermore, an increased in the urinary levels of (8-OHdG) was considered as a biomarker of oxidative stress in Down Syndrome in human subjects (Jovanovic et al., 1998), and its level was correlated with increased age in experimental animals (Drury et al., 1998; Mecocci et al., 1999; Lodovici et al., 2000), also to DNA damage in cases of Alzheimer's disease (Nunomura et al., 1999), and after nucleosides were photosensitized with dyes (Kvam et al., 1994). Also it was used as a biomarker following exposure to X and γ-rays in mice (Kasai et al., 1986).

High performance liquid chromatography (HPLC) with electrochemical detection has been used for measurement concentration of (8-OHdG) in urine (Ravanat et al., 1995; Cooke et al., 1998; Jovanovic et al., 1998; Bogdanov et al., 1999), in tissues (Floyd et al., 1986; Wiseman et al., 1995; Gedik et al., 1998; Cadet et al., 1998; Shen et al., 1999; Toraason et al., 1999; Mecocci et al., 1999; Takeuchi et al., 1999; Umemura et al., 2000), and in serum (Cooke et al., 1998). Other methods have also been used, such as gas chromatography-mass spectrometry (Wiseman et al., 1995; England et al., 1998; Evans et al., 1999; Ravanat et al., 1999); Comet assay (Gedik et al., 1998; Piperakis et al., 1999), and polyclonal antibodies (Degan et al., 1991). Recently, a method using HPLC-mass spectrometry has been developed for analysis of 8-OHdG in urine samples (Renner et al., 2000).

Permethin is a pyrethroide insecticide effective against head lice (Burgess et al., 1992), and against lyme disease (Miller, 1989). DEET is applied as an insect repellent (Brown and Hebert, 1997). Both chemicals were used by US military personnel during the Persian Gulf War to protect veterans against mosquitoes and biting insects (Young and Evans, 1998).

Permethrin modifies sodium channel to open longer during a depolarization pulse (Narahashi, 1985). Previous studies showed that DEET has direct effect on the nervous system in laboratory animals resulting in spongiform myelinopathy in the brain stem with signs include ataxia, seizures, coma and death (Verschoyle et al., 1990). Furthermore, extensive and repeated topical application of DEET resulted in human poisoning including two deaths (Roland et al., 1985; Edwards and Johnson, 1987). Combined exposure to permethrin and DEET enhanced neurotoxicity of individual chemicals in hens (Abou-Donia et al., 1996), and increased mortality in rats (McCain et al., 1997). Published reports implicated exposure to DEET and permethrin with Gulf War Illnesses (Abou-Donia et al., 1996; Haley and Kurt, 1997; Kurt, 1998; Shen, 1998; Wilson et al., 1998; Hoy et al., 2000).

No published reports examined possible effect of DEET and permethrin, alone or in combination on oxidative DNA damage. In this study, we present results of analysis of urinary levels of (8-OHdG), as a biomarker of DNA oxidative damage following a dermal dose of DEET and permethrin in rats.

2. Materials and methods

2.1. Chemicals and materials

8-Hydroxy-2′-deoxyguanosine and 2′-deoxyguanosine (Fig. 1) were obtained from Sigma Co Inc., (St. Louis, MO). DEET ($\geq$97%, *N*,*N*-diethyl-*m*-toluamide) was purchased from Aldrich Chemical Co, Inc.(Milwaukee, WIS), while permethrin (99%, 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) was obtained from Chem Service, Inc. (West Chester, PA). Waters (HPLC grade) and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Paris, Kentucky). C_{18} Sep–Pak^R Cartridges were obtained from Waters Corporation (Milford, MA).

2.2. HPLC system

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E Multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector and a Waters 2487 Dual λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2 cm × 4.0 mm, 5 μm, Supelco Park, Bellefonte, PA), and a reversed-phase C_{18} column μBondapak^{TM} C_{18} 125 Å 10 μm, 3.9 × 300 mm were used, (Waters Corporation, Milford, MA).

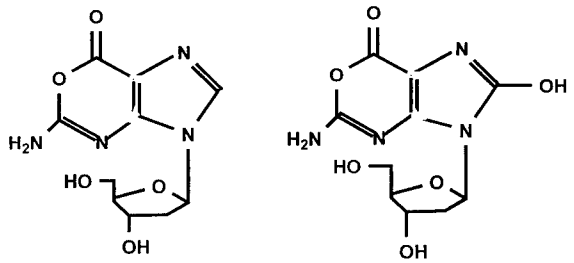

Fig. 1. Structures of 2′-deoxyguanosine to 8-hydroxy-2′-deoxyguanosine.

2.3. Calibration curve, recovery and limits of detection

Standard calibration curve of a concentration between 1–10 ng of 2′-deoxyguanosine, and 8-OHdG were obtained under the described HPLC conditions. Their detection limits were determined as the lowest concentration that can be detected taking into consideration a 1:3 base line: peak signal ratio.

Recoveries of the chemicals from urine samples were determined for concentrations of 1–10 ng. A known concentration was spiked with control urine samples (previously analyzed) and the samples were re-analyzed as described under sample preparation. Amounts of 2′-deoxyguanosine and 8-OHdG were corrected based on the recovery obtained.

2.4. Experimental animals

Sprague–Dawley rats (200–240 g) were purchased from Zivic Miller (Zelienople, PA)). The untreated animals were kept in a 12 h light/dark cycle (temperature 21–23°C) and were provided with a free supply of feed (Rodent Laboratory Chow, Ralston Purine Co., St. Louis, MO) and tap water. Animal care was conducted according to institutional guidelines.

2.5. Animals treatment

Permethrin and DEET were dissolved in 70% ethanol. A single dose of of 1.3 mg/kg of permethrin and a single dose of a 400 mg/kg of DEET were applied with a micropipette to an unprotected 1 cm^2 area of pre-clipped skin on the back of each rat. A group of five animals was used for each time point. Combined single dermal dose of 1.3 mg/kg of permethrin, followed by a single dermal dose of 400 mg of DDET was also applied. Five control rats were treated with equal volume of 70% ethanol and kept under similar conditions as treated rats.

2.6. Animals handling

After dosing, each rat was placed in a metabolic cage, and urine samples were collected at 2, 4, 8, 16, 24, 48, and 72 h after dosing. After each time point the animals were sacrificed. A 2 ml of the collected volume at each time point was taken for analysis. The samples were frozen at −20°C until analysis.

2.7. Sample preparation

A volume of 2.0 ml of the urine samples was acidified (pH 5.00) using 0.1 N acetic acid, then applied on a disposable C_{18} Sep-Pak Vac 3cc (500 mg) cartridges (Waters Corporation, Milford, MA) previously conditioned with 2 ml of methanol, and equilibrated using 2 ml of water and 2 ml of 0.03 M phosphate dibasic buffer (pH 5) prior to use. After washing with 2 ml of potassium phosphate dibasic buffer (pH 5) and 2 ml of water, the sample was eluted using 2 × 1 ml of methanol, the methanolic eluates were reduced to 500 μl using stream of nitrogen, prior to analysis by HPLC.

2.8. Analysis

A volume of 10.0 μl of the extracts was injected into the HPLC system as described above. The mobile phase consisted of 83% water (adjusted to pH 3.00 using 0.1 M acetic acid), and 17% acetonitrile at flow rate of 0.50 ml/min. The eluents were monitored by UV detection at 254 nm. The chromatographic analysis was performed at ambient temperature. Amount of 8-Hydroxy-2′-deoxyguanosine and 2′-deoxyguanosine was calculated and corrected for the urine volume and total body weight of the animal.

2.9. Statistical analysis

Analysis of variance using GrphPad Prism program for Windows (GraphPad Software, Inc., San Diego, CA) was used to determine if the difference between treated and control is significant.

3. Results

3.1. Linearity, recovery and detection limits

Calibration standard curves for 2′-deoxyguanosine and 8-OHdG was obtained for a concentration ranged between 1–10 ng (Fig. 2). Recovery of 2-deoxyguanosine and 8-hydroxy-2-deoxyguanosine from urine samples was determined for concentrations ranged between 1–100 ng. Average percentage recoveries were 84.8 ± 9.2% to 75.5 ± 6.8% for 2′-deoxyguanosine and 8-hydroxy-2′-deoxyguanosine (8-OHdG), respectively. Limits of detection of 2-deoxyguanosine and 8-hydoxy-2-deoxyguanosine were 0.5 ng.

3.2. HPLC analysis

Fig. 3 and Fig. 4 show the chromatograms of standard and urine samples of 2′-deoxyguanosine and 8-hydroxy-2′-deoxyguanosine under described HPLC conditions. Retention times were 10.7 min, and 12.6 min for 2′-deoxyguanosine and 8-hydroxy-2′-deoxyguanosine, respectively.

3.3. Levels of 2-deoxyguanosine and 8-hydroxy-2-deoxyguanosine

A single dermal dose of 400 mg/kg of DEET caused significant increase in the urinary excretion of 8-hydroxy-2′-deoxyguanosine when applied, alone or in combination with a dermal dose of 1.3 mg/kg of permethrin. Amount of 8-hydroxy-2′-deoxyguanosine and 2′-deoxyguanosine was corrected according to total volume of the urine and body weight of the animal. A single dermal dose of 1.3 mg/kg of permethrin produced an increase in the levels of 8-hydroxy-2′-deoxyguanosine in rat urine that was not statistically significant (Fig. 5 and Fig. 6). Level of 8-hydroxy-2′-deoxyguanosine and 2′-deoxyguanosine was calculated based on the percentage recovery of each chemical.

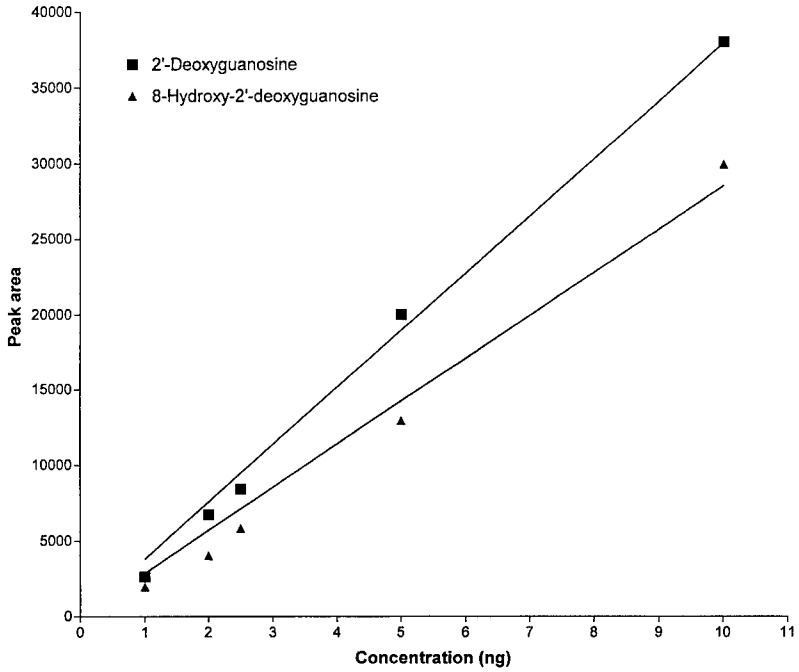

Fig. 2. Standard calibration curves of 2′-deoxyguanosine and 8-hydroxy-2′-deoxyguanosine.

3.4. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine if application of DEET and permethrin, alone or in combinations has caused significant increase in the levels of 8-OHGd excreted in the urine. DEET caused significant induction of 8-OHdG urinary levels ($P < 0.05$), while there was no significant difference of permethrin treatment and ethanol treated rats, or between DEET alone and DEET + permethrin treatment ($P < 0.1$). Prism program for Windows (GraphPad Software, Inc., San Diego, CA) was used for the analysis.

4. Discussion

Our results indicate that dermal administration of DEET to rats induced oxidative DNA damage as shown by increased urinary excretion of 8-hydroxy-2′-deoxyguanosine (8-OHdG) following DEET application. Both dermal doses of DEET when applied alone or in combination with permethrin caused similar induction. It seems that DEET caused free radical generation following application in rats, thus increasing the urinary levels of 8-OHdG compared to control urine samples. In this study we used a real-life exposure levels as determined by US Department of Defense (Personal Communications).

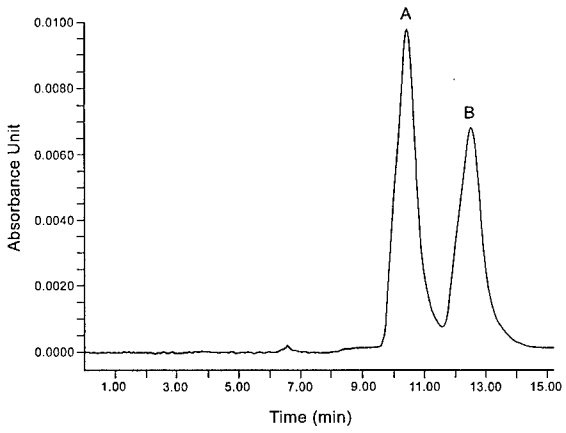

Fig. 3. HPLC chromatogram of standard (A) 2′-deoxyguanosine, (B) 8-hydroxy-2′-deoxyguanosine.

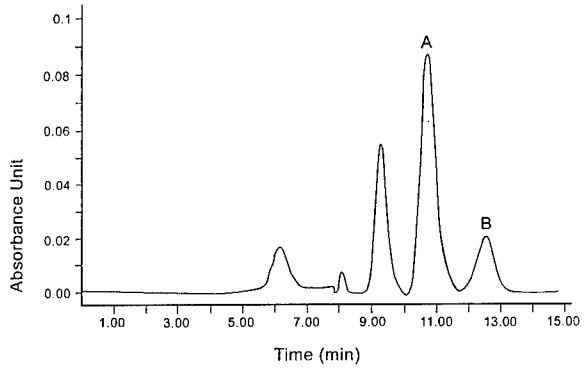

Fig. 4. HPLC chromatogram of urine sample of rats treated with a single dermal dose of 400 mg/kg of DEET (A) 2′-deoxyguanosine, (B) 8-hydroxy-2′-deoxyguanosine.

Although HPLC with UV detection is not the most sensitive method, separation of 8-OHdG from 2′-deoxyguanosine was achieved under the described HPLC condition. Our limit of detection was 0.5 ng compared to the more sensitive HPLC-MS, with detection of 8-OHdG in human urine of 0.2 ng/ml (Renner et al., 2000). Our ability to detect both chemicals in most samples analyzed (2 ml urine) proved that our method is adequate in assessing DNA oxidative damage by measuring levels of 8-OHdG as a biomarker. The induction of urinary excretion of 8-OHdG in our experiment is consistent with previous studies following exposure to chemicals. Wellejus et al. (2000) reported increased of 8-OHdG excretion rate from 129 to147 pmole 24 h after iv dose of iron in rats, and the urinary excretion 8-OHdG was significantly higher in rats received a single i.p. dose of 100 mg/kg of 2-nitropropane (Toraason et al., 1999). Also Yusof et al., (1999) reported that δ-aminolevulinic acid caused linear increase in 8-OHdG levels in Chinese hamster ovary, while incubation of o-phenylphenol metabolites, o-phenylhydroquinone and o-phenylbenzoquinone with Chinese hamster cells significantly enhanced of 8-OHdG concentration in nuclear DNA (Henschke et al., 2000). Similar finding was also reported following administration of 4 mg cupper/kg rat that caused significant increased of 8-OHdG levels of DNA liver and kidney

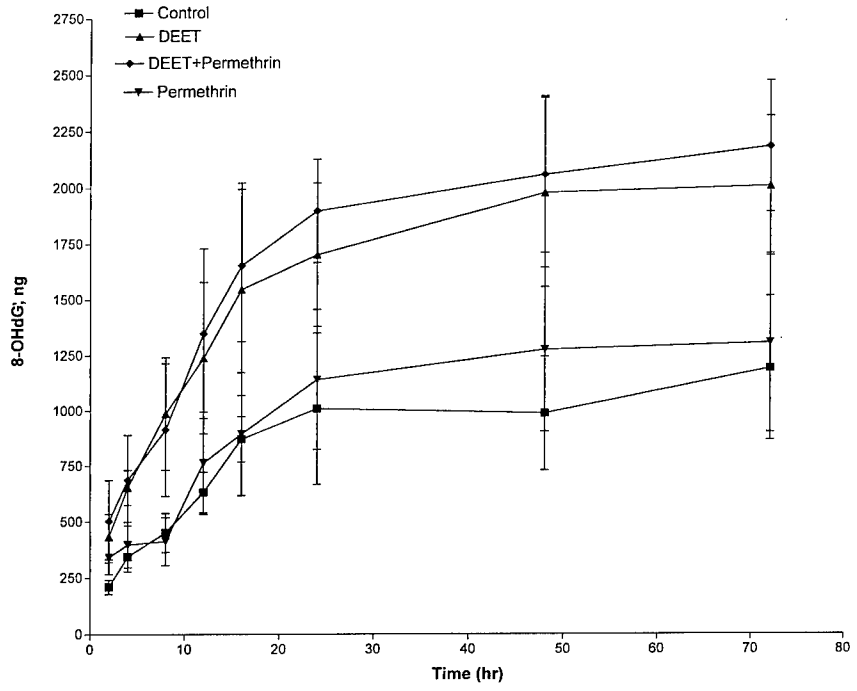

Fig. 5. Levels of 8-hydroxy-2′-deoxyguanosine in urine samples following a single dermal dose of DEET, and permethrin, alone and in combination.

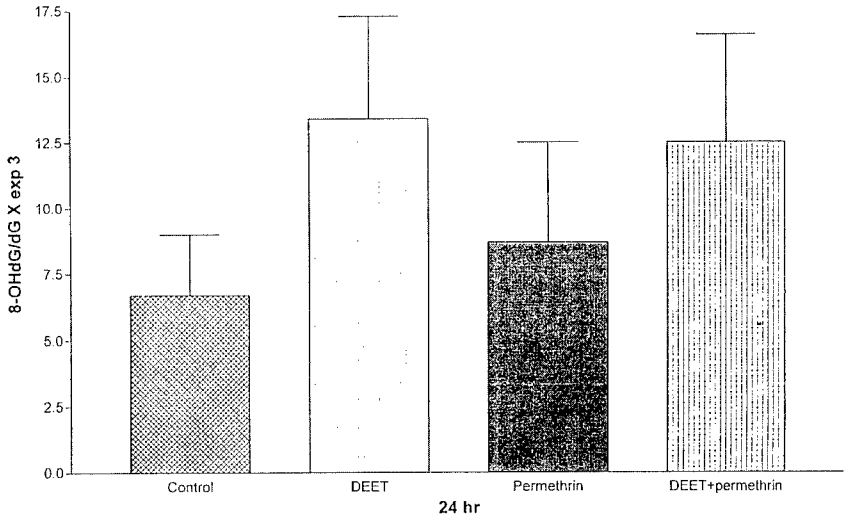

Fig. 6. Excretion of 2′-deoxyguanosine and 8-hydroxy-2′-deoxyguanosine in urine samples collected 24 h after a single dermal dose of DEET and permethrin, alone and in combination.

(Toyokuni and Sagripanti, 1994). Addition of dimethylformamide enhanced about 3–4 fold 8-OHdG formation induced by H_2O_2 and cupper in calf thymus DNA (Midorikawa et al., 2000). Sim-ilar results were obtained in other species; exposure of fish to the insecticide dieldrin increased 8-OHdG levels of DNA liver (Rodriguez-Ariza et al., 1999), an increased in the amount of 8-OHdG was reported in diseases causing DNA oxidative damage. Jovanovic et al., (1998) detected about two fold increase in urinary excretion of 8-OHdG in 166 individuals with Down Syndrome compared to healthy subjects, and its levels were significantly higher in human sperm of infertile patients compared to healthy subjects (Shen et al., 1999). Other factors also increased 8-OHdG levels, such as in human leukocytes of smokers where the amount of 8-OHdG was significantly higher compared to non-smokers (Lodovici et al., 2000).

Urinary excretion of 8-OHdG reached a peak level after 24 h of dermal dose of DEET alone, or in combination with permethrin, then gradually leveled off. Peak levels of 8-OHdG in the excreted urine following treatment with DEET were 1897 ng/24h. Loft et al., (1993) reported a range between 200–300 pmol/kg/24h of 8-OHdG in the urine of 169 humans where smokers excreted 50% higher than non-smoker subjects. Also Loft et al., (1998b) detected a levels between 400–2000 pmol/kg rat, that depended on type of diet.

Our results show that exposure of rats to a dermal dose of 1.3 mg/kg of permethrin did not cause significant increase of urinary excretion of 8-OHdG, suggesting that oxidative DNA damage was similar to that of control levels. The small dermal dose of 1.3 mg/kg of permethrin used and its slow dermal absorption compared to DEET might have resulted in a small concentration below the threshold level to reach the DNA target. No published data reported that pyrethroides significantly increased free radical formation. Umemura et al. (2000) reported no increased concentration of 8-OHdG levels in the kidney nuclear DNA following sub-chronic exposure of rats to *p*-dichlorobenzene. In summary, dermal application of DEET, alone or in combination with dermal dose of permethrin increased levels of 8-OHdG in rats urine.

Acknowledgements

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Appendix 6
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Mohamed B. Abou-Donia

Locomotor and Sensorimotor Performance Deficit in Rats following Exposure to Pyridostigmine Bromide, DEET, and Permethrin, Alone and in Combination

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Since their return from Persian Gulf War (PGW), many veterans have complained of symptoms including muscle and joint pain, ataxia, chronic fatigue, headache, and difficulty with concentration. The causes of the symptoms remain unknown. Because these veterans were exposed to a combination of chemicals including pyridostigmine bromide (PB), DEET, and permethrin, we investigated the effects of these agents, alone and in combination, on the sensorimotor behavior and central cholinergic system of rats. Male Sprague-Dawley rats (200–250 gm) were treated with DEET (40 mg/kg, dermal) or permethrin (0.13 mg/kg, dermal), alone and in combination with PB (1.3 mg/kg, oral, last 15 days only), for 45 days. Sensorimotor ability was assessed by a battery of behavioral tests that included beam-walk score, beam-walk time, incline plane performance, and forepaw grip on days 30 and 45 following the treatment. On day 45 the animals were sacrificed, and plasma and CNS cholinesterase, and brain choline acetyl transferase, muscarinic and nicotinic acetylcholine receptors were evaluated. Animals treated with PB, alone or in combination with DEET and permethrin, showed a significant deficit in beam-walk score as well as beam-walk time as compared with controls. Treatment with either DEET or permethrin, alone or in combination with each other, did not have a significant effect on beam-walk score. All chemicals, alone or in combination, resulted in a significant impairment in incline plane testing on days 30 and 45 following treatment. Treatment with PB, DEET, or permethrin alone did not have any inhibitory effect on plasma or brain cholinesterase activities, except that PB alone caused moderate inhibition in midbrain acetylcholinesterase (AChE) activity. Treatment with permethrin alone caused significant increase in cortical and cerebellar AChE activity. A combination of DEET and permethrin or PB and DEET led to significant decrease in AChE activity in brainstem and midbrain and brainstem, respectively. A significant decrease in brainstem AChE activity was observed following combined exposure to PB and permethrin. Coexposure with PB, DEET, and permethrin resulted in significant inhibition in AChE in brainstem and midbrain. No effect was observed on choline acetyl transferase activity in brainstem or cortex, except combined exposure to PB, DEET, and permethrin caused a slight but significant increase in cortical choline acetyltransferase activity. Treatment with PB, DEET, and permethrin alone caused a significant increase in ligand binding for m2 muscarinic acetylcholine receptor (mAChR) in the cortex. Coexposure to PB, DEET, and permethrin did not have any effect over that of PB-induced increase in ligand binding. There was no significant change in ligand binding for nicotinic acetylcholine receptor (nAChR) associated with treatment with the chemical alone; a combination of PB and DEET or coexposure with PB, DEET, and permethrin caused a significant increase in nAChR ligand binding in the cortex. Thus, these results suggest that exposure to physiologically relevant doses of PB, DEET, and permethrin, alone or in combination, leads to neurobehavioral deficits and region-specific alterations in AChE and acetylcholine receptors.

Key Words: Persian Gulf War; sensorimotor; pyridostigmine bromide; DEET; permethrin; combined exposure; CNS.

Since their return from the war, many Persian Gulf War (PGW) veterans have complained of symptoms including chronic fatigue, muscle and joint pain, ataxia, rash, headache, difficulty concentrating, forgetfulness, and irritability (Institute of Medicine, 1995). Haley *et al.* (1997a,b), used epidemiological analyses to characterize these symptoms into six syndromes. The veterans in the PGW were exposed to a unique combination of biological, chemical, and psychological environments. Combinations of chemical exposures included a variety of pesticides such as DEET and permethrin (Institute of Medicine, 1995). Additionally, these veterans were given a course of twenty-one 30-mg tablets of pyridostigmine bromide (PB) as prophylactic treatment to protect against organophosphate (OP) nerve agents (Persian Gulf Veterans Coordinating Board, 1995). PB is viewed to be relatively safe at the given dose.

PB is a quaternary dimethyl carbamate used as a treatment for myasthenia gravis at a higher dose range than what was

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given to PGW veterans (Breyer-Pfaff *et al.,* 1985, 1990). PB reversibly inhibits 30–40% of the AChE in the peripheral nervous system, thus limiting irreversible inhibition of the enzyme by nerve agents (Blick *et al.,* 1991). AChE activity is restored following spontaneous decarbamylation resulting in near-normal neuromuscular and autonomic functions (Blick *et al.,* 1991). Toxic symptoms associated with PB overdose results from overstimulation of nicotinic and muscarinic receptors in the peripheral nervous system, resulting in exaggerated cholinergic effects such as muscle fasciculations, cramps, weakness, muscle twitching, tremor, respiratory difficulty, gastrointestinal tract disturbances, and paralysis (Abou-Donia *et al.,* 1996; McCain *et al.,* 1997). With severe intoxication, death may occur because of asphyxia. Central nervous system effects of PB are not expected unless BBB permeability is compromised. The positive charge on the quaternary pridinyl nitrogen prevents PB from crossing the intact BBB (Birtley *et al.,* 1966).

The insect repellent *N,N*-diethyl-*m*-toluamide (DEET) and the insecticide pyrethroid permethrin 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl) methyl ester have been used extensively by humans since their introduction. DEET is commonly used as a repellant against mosquitoes, flies, ticks, and other insects (McConnel *et al.,* 1986; Robbins and Cherniack, 1986). However, extensive and repeated topical DEET applications can cause human poisoning, including death (Edwards and Johnson, 1987; Gryboski *et al.,* 1961; Roland *et al.,* 1985). The symptoms associated with DEET poisoning include tremors, restlessness, difficulty with speech, seizures, impairment of cognitive function, and coma (McConnel *et al.,* 1986). DEET efficiently crosses the dermal barrier and may localize to dermal fat deposits (Blomquist and Thorsell, 1977: Snodgrass *et al.,* 1982). Although the exact mechanisms of DEET toxicity are not known, extremely high levels of DEET exposure cause demyelination and spongiform myelinopathy in the rat (Verschoyle *et al.,* 1992).

Permethrin is a type I synthetic pyrethroid insecticide that exists in four different stereoisomers (Casida *et al.,* 1983). It provides insecticidal activity for several weeks following a single application and is used in a variety of public buildings, industrial premises, and private dwellings to control fleas, flies, mites, and cockroaches. Permethrin intoxication results as a consequence of modification of sodium channels, leading to prolonged depolarization and repetitive discharges in presynaptic nerve fibers after a single stimulus (Narahashi, 1985). This repetitive nerve action is associated with tremor, hyperactivity, ataxia, convulsions, and in some cases to paralysis. Permethrin is detoxified by ester hydrolysis in the blood and most tissues.

We previously reported that concurrent exposure to relatively large doses of PB, DEET, and permethrin in hens resulted in neurotoxic effects greater than those produced by exposure to the single components (Abou-Donia *et al.,* 1996). In the present study we have extended these observations to include doses similar to levels of human exposure. We evaluated whether PB would enhance the neurotoxic effects caused by low-dose, combined exposure to DEET and permethrin. These results suggest that treatment with PB, DEET, and permethrin, alone or in combination, causes a significant impairment in sensorimotor abilities and region-specific effects on brain AChE and mAChR.

MATERIALS AND METHODS

Chemicals. Technical-grade (≥ 93.6%) permethrin 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl) methyl ester was obtained from Roussel Uelaf Corp., Pasadena, TX. DEET (99.7% *N,N*-diethyl-*m*-toluamide), pyridostimine bromide (≥ 99%, 3-dimethylamino carbonyloxy-*N*-methylpridinium bromide), acetylthiocholine iodide, butyrylthiocholine iodide, atropine, and nicotine were purchased from Sigma Chemical Co., St. Louis, MO. 5,5′–dithio-bis-2-nitrobenzoic acid (DTNB) was purchased from Aldrich. The inhibitor, 1,5-bis-(*N*-allyl-*N,N*-dimethyl-4-ammonium phenyl) pentan-3-one dibromide (BW284C51) was obtained from Sigma Chemical Co, St. Louis MO. [^{3}H]AF-DX 384, sp. activity 106 μCi/mmol, [^{3}H]cytisine, sp. activity 32 nCi/pmol, and [^{3}H]acetyl CoA, sp. activity 12 μCi/mmol were purchased from New England Nuclear, Boston, MA. All other chemicals and reagents were of highest purity available from commercial sources.

Animals. Male Sprague-Dawley rats weighing 225–250 g were obtained from Zivic-Miller Laboratories, Allison Park, PA, and housed at Duke University Medical Center vivarium. The animals were randomly assigned to control and treatment groups and housed at 21–23°C with a 12-h light/dark cycle. They were supplied with food and water *ad libitum.* The rats were allowed to adjust to their environment for a week before starting the treatment. Animal care was in accordance with institutional guidelines.

Treatment. The dosages of chemicals used were as follows: PB (1.3 mg/kg/day in water, oral), DEET (40 mg/kg/day in 70% ethanol, dermal), and permethrin (0.13 mg/kg/day in 70% ethanol, dermal). These doses of PB, DEET, and permethrin correspond to real-life exposure to military personnel during the PGW. Oral doses were given by gavage, while dermal applications were applied to the back of the neck on a 1-in^2 area preshaved with electric clippers. Groups with five animals each were treated as described below. Animals were sacrificed 24 h after treatment with the last dose.

- Control: animals receiving daily dermal dose of 70% ethanol and water for the last 15 days of the experiment
- Pyridostigmine bromide for the last 15 days of the experiment
- DEET daily for 45 days
- Permethrin daily for 45 days
- DEET and permethrin daily for 45 days
- DEET for 30 days, then PB + DEET for the last 15 days
- Permethrin for 30 days, then PB + permethrin for the last 15 days
- DEET and permethrin for 30 days, then PB + DEET + permethrin for the last 15 days

Behavioral Studies. A battery of standardized tests was employed on days 30 and 45 following the treatment. These behavioral tests were designed to measure sensorimotor reflexes, motor strength, and coordinated gait (Bederson *et al.,* 1986; Goldstein, 1993; Markgraf *et al.,* 1992). All behavioral testing was performed by a trained observer blind to the treatment status of the animal and was carried out in a soundproof room with subdued lighting (less than 10.76 lumens/m^2, ambient light). Rats were handled for 2 min daily for 5 days during the week prior to behavioral testing.

Postural Reflexes

Description. Rats were held gently by the tail, one meter above the floor, and observed for forelimb extension. Normal rats extend both forelimbs.

Consistent flexion of the forelimb is an abnormal response. Rats with consistent forelimb flexion are then further assessed by being placed on a large sheet of plastic-coated paper that can be gripped with the forepaws. With the tail held, gentle lateral pressure was applied behind the shoulder of the rat until the forelimb slid several inches. The maneuver was repeated five times in each direction. Normal rats resist lateral pressure by gripping the coated paper.

Scoring. Grade 0: rats without evidence of consistent forelimb flexion when held above the floor; grade 1: rats with consistent forelimb flexion; grade 2: otherwise grade 1 rats that do not resist lateral pressure on at least three of five trials in either direction.

Limb Placing

Description. Visual, tactile, and proprioceptive forelimb placing responses were examined. For visual placing, rats were held in the hands of the examiner 10 cm above the tabletop, with forelimbs hanging free. The rats were then slowly tilted toward the table. Intact rats reach toward the table with both forepaws. For tactile placing, the dorsal and then lateral portions of the forepaws were touched to the table edge. Intact rats immediately place the paw on the surface of the table. Proprioceptive placing was tested by pushing the forepaw onto the table edge. Care was taken to avoid the vibrissae touching the table.

Scoring. For each test: grade 0, the placing response is immediate; grade 1, the placing response is slow or delayed; grade 2, the placing response does not occur within 2 s.

Orienting to Vibrissae Touch

Description. The rat was placed atop an inverted polycarbonate cage and allowed 1 min for habituation. Its vibrissae were then touched with a cotton-tipped swab.

Scoring. Grade 0: rat orients to the side of the probe on at least two of three trials from each side; grade 1, rat fails to orient on at least two of three trials on either side.

Grip Time. Forepaw grip time of the rats was assessed by having them hang from a 5 mm diameter wood dowel gripped with both forepaws. Time to release their grip was recorded in seconds.

Beam-Walking and Beam Score

Description. The testing apparatus was a 2.5 × 122 cm wooden beam elevated 75.5 cm above the floor with wooden supports. A 20 × 25 × 24 cm goal box with a 9.5 cm opening is located at one end of the beam. A switch-activated source of bright light (75 watt Tungsten bulb) and white noise (41 dB at 8000 Hz, 58 dB at 4000 Hz, 56 dB at 2000 Hz, 56 dB at 1000 Hz, 58 dB at 500 Hz, and 52 dB at 250 Hz SPL at the center of the frequency at each octave band) were located at the start end of the beam and served as avoidance stimuli. The rats were first trained with a series of three approximate trials. Rats are readily trained to perform the beam-walking task (Goldstein, 1993).

Scoring. Both the latency until the animal's nose entered the goal box (up to 90 s) and the use of the hind paw to aid locomotion were recorded. Beam-walking ability was measured with a seven-point scoring system scale as previously described (Goldstein, 1993): 1, the rat is unable to place the affected hindpaw on the horizontal surface of the beam; 2, the rat places the hindpaw on the horizontal surface of the beam and maintains balance for at least 5 s; 3, the rat traverses the beam while dragging the affected hindpaw; 4, the rat traverses the beam and at least once places the affected hindpaw on the horizontal surface of the beam; 5, the rat crosses the beam and places the affected hindlimb on the horizontal surface of the beam to aid less than half its steps; 6, the rat uses the affected hindpaw to aid more than half its steps; and 7, the rat traverses the beam with no more than two footslips. In addition, the latency until the animal's nose enters the goal box (up to 90 s) is recorded for the final trial. Rats that fell off the beam were assigned latencies of 90 s.

Incline Plane

Description. The rats were placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised according to the method described by Yonemori *et al.* (1998). The board was slowly rotated to the vertical position. Two trials were performed for each testing session.

Scoring. The angle that the rat began to slip downward was recorded. The results of two trials were averaged at each time point.

Statistical Analysis. For continuous data (beam-walk time, beam-walk score, grip time, and incline plane), groups were compared by two-way ANOVA, with repeated measures as appropriate. The significance of post hoc pairwise comparison was determined with Fisher's LSD tests. For nonparametric data (postural reflexes, limb placing, and vibrassal touch), comparisons across treatment groups were made with the Kruskal-Wallis test. If the Kruskal-Wallis test indicated a significant difference among the groups, Dunn's procedure would be applied to the ranks of the data to determine the significance of post hoc, pairwise comparisons.

Assays

Acetylcholinesterase and butyrylcholinesterase assays. Brain acetylcholinesterase (AChE) and plasma cholinesterase (BChE) activities were measured by the Ellman assay (Ellman *et al.*, 1961). For AChE assays, dissected brain regions were homogenized in Ellman buffer and centrifuged for 5 min at 5000 × *g*; the resulting supernatant was used for AChE analysis. AChE activity was measured using acetylthiocholine as substrate in a Molecular Devices UV Max Kinetic Microplate Reader at 412 nm. 5,5′-Dithio-bis-2-nitrobenzoic acid (DTNB) was used as the color reagent as described by Abou-Donia *et al.* (1996). Protein concentrations in tissue samples and plasma were determined by the method of Smith *et al.* (1985).

Choline acetyl transferase. Choline acetyl transferase activity in brain was determined using methods by Fonnum (1975).

Muscarinic acetylcholine receptor binding. For the assay of the ligand binding for m2 mAChR, the tissue was homogenized in 10 mM phosphate buffer, pH 7.4, and centrifuged at 40,000 × g for 10 min. The membranes were suspended in the same buffer at a protein concentration of 1.25–2.5 mg/ml as described by Huff *et al.* (1994), and the ligand binding was carried out according to Slotkin *et al.* (1999). The m2 mAChR binding was carried out by using m2 mAChR-specific ligand, [^{3}H]AF-DX 384 at room temperature for 60 min. Results are presented as specific receptor binding (dpm)/mg protein (percent of control).

Nicotinic acetylcholine receptor binding. [^{3}H]Cytisine was used as specific ligand for nAChR according to the method described by Slotkin *et al.* (1999). An aliquot of membrane preparation containing ~200 μg protein was used to carry out the incubation with 1 nM [^{3}H]cytisine at 4°C for 75 min. Results are presented as specific receptor binding (dpm)/mg protein (percent of control).

Statistics. For biochemical assays, treatment groups were compared to control groups by two-way unpaired *t*-test using Prism GraphPad™ software, and results were plotted using Excel graphics for Macintosh.

RESULTS

General Health and Clinical Condition

There were no overt clinical signs of toxicity observed throughout the study except for occasional diarrhea in rats receiving DEET. There were no significant differences in weights between the treatment groups throughout the study.

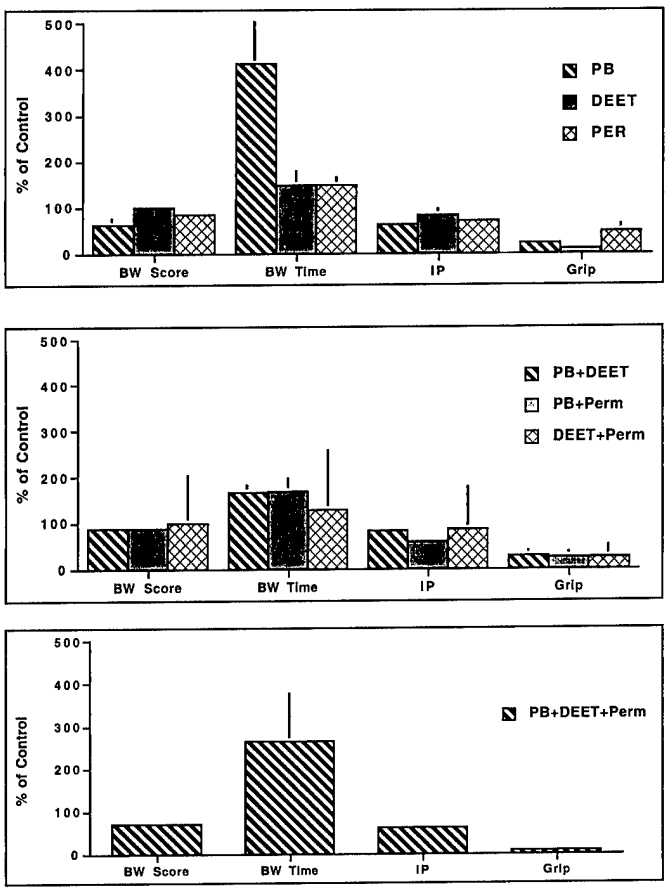

FIG. 1. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on sensorimotor performance on day 30 of the beginning of treatment with DEET or permethrin. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. The animals were examined blindfolded for beam-walk score (BW score), beam-walk time (BW time), incline plane (IP), and grip response. The data were computed and detailed statistical evaluations were carried out as described in the Results section. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. For comparison purposes, the data are presented as means ± SE (percent of control).

Effect of DEET, Permethrin or PB, Alone or in Combination, on Sensorimotor Function

A battery of behavioral tests was carried out to assess the sensorimotor function. We focused on sensorimotor ability because the majority of PGW veterans' complaints related to muscle and joint pain, fatigue, disorientation, and ataxia. Animals were tested on days 30 and 45 from the beginning of the treatment. Although statistical analyses were performed on the actual data, for the sake of comparability, the data obtained from beam-walk score, beam-walk time, incline plane, and grip time are presented as a percent of control. In the figures, error bars reflect SEM based on the raw data and recalculated to reflect percent of control. Figure 1 represents the measurements carried out on day 30, and Figure 2 represents the activity measured on day 45 following the beginning of the treatment with DEET and permethrin.

There was no effect of any of the drugs, alone or in combination, on postural reflexes, limb placing, or vibrissae touch (data not shown). Control animals consistently showed completely normal performance (Kruskal-Wallis, $p > 0.05$ for each comparison). For beam-walk score, two-way repeated measures ANOVA showed a significant effect of treatment group (ANOVA $F_{7,70} = 8.4$, $p < 0.0001$) and a significant treatment group × time interaction effect (ANOVA $F_{1,7} = 2.3$, $p = 0.03$). Given alone, PB, but not DEET or permethrin, differed significantly from control (Fisher LSD, $p = 0.0001$). The poorest performance was seen in rats that received all three drugs and

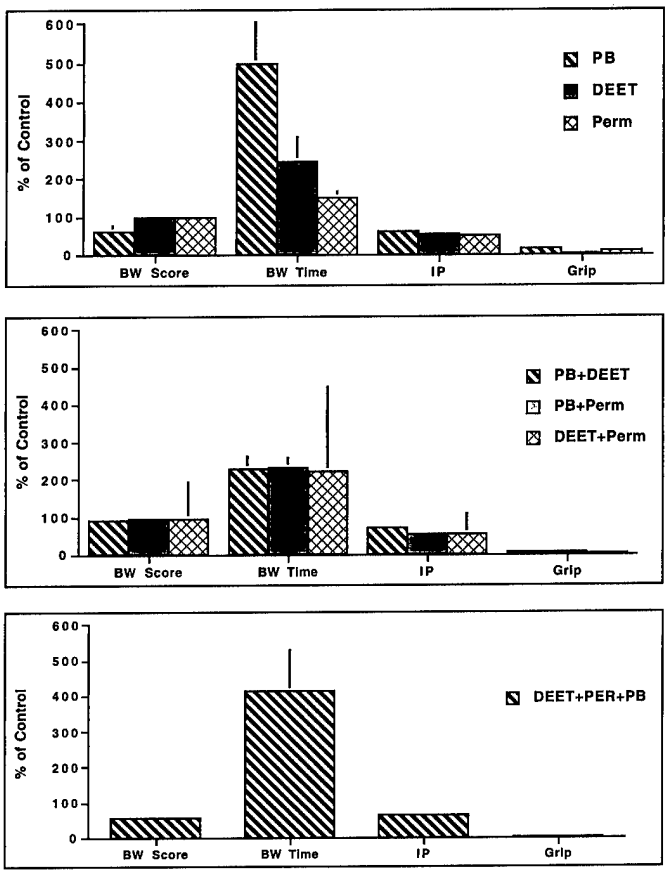

FIG. 2. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on sensorimotor and locomotor performance on day 45 of the beginning of treatment with DEET or permethrin. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. The animals were examined blindfolded for beam-walk score (BW score), beam-walk time (BW time), incline plane (IP), and grip response. The data were computed and detailed statistical evaluations were carried out as described in the Results section. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. For comparison purposes, the data are presented as means ± SE (percent of control).

those receiving PB. There was no significant difference in performance between rats given PB and those given DEET, permethrin, and PB (Fisher LSD, $p = 0.71$).

For beam-walking time, two-way repeated measures ANOVA showed a significant effect of treatment group (ANOVA $F_{7,70} = 8.4$, $p = 0.006$) but no treatment group × time interaction (ANOVA $F_{1,7} = 0.7$, $p = 0.65$). Given alone, pyridostigmine, but not DEET or permethrin, differed significantly from control (Fisher LSD, $p = 0.0002$). There was not a significant difference between rats given PB and those given DEET, permethrin, and PB (Fisher LSD, $p = 0.20$).

For incline plane performance (Figs. 1 and 2), two-way repeated measures ANOVA showed a significant effect of treatment group (ANOVA $F_{7,70} = 38.1$, $p < 0.0001$) and a significant treatment group × time interaction (ANOVA $F_{1,7} = 4.5$, $p = 0.004$). All drugs given alone differed significantly from control (Fisher LSD, $p < 0.0001$). There was no significant difference between rats given PB and those given DEET, permethrin, and PB (Fisher LSD, $p > 0.9$).

Finally, for forepaw grip time (Figs. 1 and 2), two-way repeated measures ANOVA showed a significant effect of treatment group (ANOVA $F_{7,70} = 44.5$, $p = 0.001$) and a significant treatment group × time interaction (ANOVA $F_{1,7} = 4.9$, $p = 0.001$). All drugs given alone differed significantly from control (Fisher LSD, $p < 0.0001$). There was no significant difference between rats given PB and those given DEET, permethrin, and PB (Fisher LSD, $p = 0.06$).

A similar decreasing but insignificant trend in horizontal and vertical movement was observed in the animals treated with the combination of PB, DEET, and permethrin (data not shown).

In summary, each drug given alone had a significant behavioral effect, which tended to become more evident over time. There also was no significant difference on any parameter between rats given PB alone and those given DEET, permethrin, and PB. Most significant deficits were observed in animals given PB or a combination of PB with other chemicals.

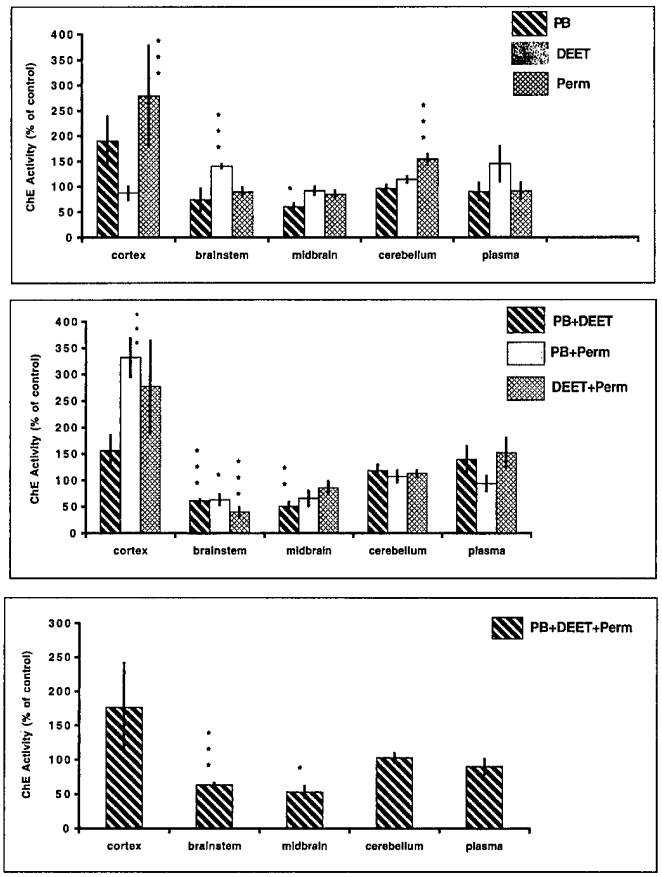

FIG. 3. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on brain regional AChE and plasma BChE activities. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. Treatment with PB was given on the last 15 days of the experiment. The details of the treatment and determination of enzyme activity are elaborated in Materials and Methods. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. Data are presented as means ± SE (percent of control). p value: * < 0.04, ** < 0.01, *** < 0.001.

Effect of PB, DEET, and Permethrin, Alone or in Combination, on Plasma and Brain Cholinesterase Activities

Plasma BChE and AChE activities in cortex, brainstem, midbrain, and cerebellum from the animals treated with PB, DEET, and permethrin, alone or in combination, were assayed. Data on the effects of single-chemical treatment are presented in Figure 3 (top panel). Treatment with PB, alone or in combination with DEET and permethrin, caused slight but insignificant inhibition (~ 96% of controls) of plasma BChE activity. Treatment with DEET, alone or in combination with PB or permethrin, caused a variable but insignificant increase (~ 125–140% of control) in BChE activity. Treatment with permethrin, either alone or in combination, did not have any effect on BChE activity.

Treatment with PB alone inhibited the AChE activity in midbrain (~ 60% of control, $p < 0.04$) and produced no significant changes in enzyme activity in brainstem, cortex, and cerebellum. DEET or permethrin treatment alone did not cause any significant inhibitory effect on brain region AChE activities. Instead, DEET treatment alone caused a significant increase (~ 140% of control) in brainstem enzyme activity, and permethrin alone caused a significant increase (~ 278% of control) in cortical enzyme activity ($p < 0.001$).

Data on combination of two chemicals are presented in Figure 3 (middle panel). A combination of PB and DEET treatment resulted in significant AChE inhibition in the brainstem and midbrain ($p < 0.001$ and 0.01, respectively). Combined exposure with PB and permethrin resulted in significant inhibition (~ 67% of control, $p < 0.04$) in brainstem AChE activity, whereas cortex activity remained significantly increased. Combined exposure with DEET and permethrin re-

sulted in significant inhibition ($p < 0.001$) in brainstem AChE activity, whereas other regions did not show any change in the activity that was different than individual chemical alone. This was consistent with our previous studies (Abou-Donia *et al.*, 1996).

Data presented in Figure 3 (bottom panel) indicate that brainstem and midbrain AChE activity was significantly inhibited when animals were exposed to the combination of PB, DEET, and permethrin (~ 60–65% of control, $p < 0.001$ and 0.04, respectively). The magnitude of inhibition is similar to that observed when animals were exposed to a combination of PB and DEET, suggesting that PB under these treatment conditions has the potential to inhibit the brainstem and midbrain AChE activity.

Effect of PB, DEET, and Permethrin, Alone or in Combination, on Brain Choline Acetyl Transferase (ChAT) Activity

Choline acetyl transferase (ChAT) catalyzes the final step in the biosynthesis of acetylcholine by facilitating the irreversible transfer of acetyl groups of acetylCoA to choline. In view of the changes induced by PB in CNS on AChE activity, and because PB-induced inhibition of AChE is reversible and short-lived (Watts and Wilkinson, 1977), we argued that there may exist alternative mechanisms of acetylcholine buildup. Therefore, we studied the effects of treatment with PB, DEET, and permethrin, alone or in combination, on ChAT activity in brainstem and cortex. In the CNS, the majority of ChAT activity is localized in brainstem and cortex (Wu and Hersh, 1994). Data in Figure 4 represent the enzyme activity in the cortex and brainstem. Treatment with PB and permethrin alone did not have any significant effect on brainstem or cortex enzyme activities, whereas DEET treatment caused a significant increase in the enzyme activity in the cortex ($p < 0.001$). Combined exposure of PB and permethrin caused a significant increase ($p < 0.001$) in ChAT activity in the cortex. However, coexposure with PB, DEET, and permethrin did not result in any significant change in enzyme activity in either region.

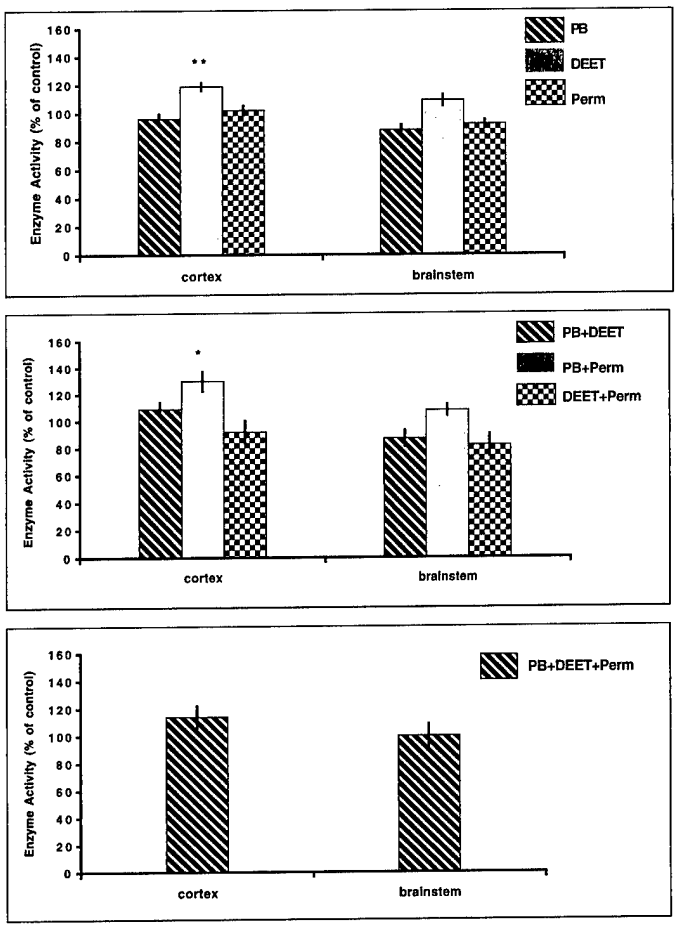

FIG. 4. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on cortex and brainstem choline acetyl transferase activity. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. Treatment with PB was given on the last 15 days of the experiment. The details of the treatment and determination of enzyme activity are elaborated in Materials and Methods. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. Data are presented as means ± SE (percent of control). p value: * < 0.01, ** < 0.001.

Effect of PB, DEET, and Permethrin, Alone or in Combination, on m2 Muscarinic and Nicotinic Acetylcholine Receptor Activity

In order to evaluate the effect of treatment with PB, DEET, and permethrin, alone or in combination, on muscarinic receptor, ligand binding studies were carried out with membrane preparations using m2-specific ligand [^{3}H]AFDX in cortex, brainstem, midbrain, and cerebellum. The data presented in Figure 5 indicate that PB treatment alone caused a significant increase in ligand binding density in the cortex (~ 165% of control, $p < 0.001$) and no effect in midbrain and brainstem. Treatment with DEET or permethrin alone caused a significant increase in ligand binding density in the cortex. A similar increase in ligand binding in the cortex was observed with combined exposure of PB and DEET, and DEET and permethrin. There was a significant increase (~ 150%, $p < 0.006$) in cerebellum in the animals treated with PB and permethrin. Combined exposure with PB, DEET, and permethrin led to significant increase in ligand binding only in cortex ($p < 0.001$).

Ligand binding for nicotinic acetylcholine receptors using [^{3}H]cytisine was carried out in the cortex membranes prepared from the animals treated with PB, DEET, and permethrin, alone or in combination. The data presented in Figure 6 show that treatment with PB, DEET, and permethrin alone did not cause any significant change in ligand binding. Treatment with DEET in combination with PB or permethrin led to a significant increase in the ligand binding density (~ 125% of control. $p < 0.03$). Coexposure with PB, DEET, and permethrin caused a significant increase (~ 138%, $p < 0.03$) in ligand binding.

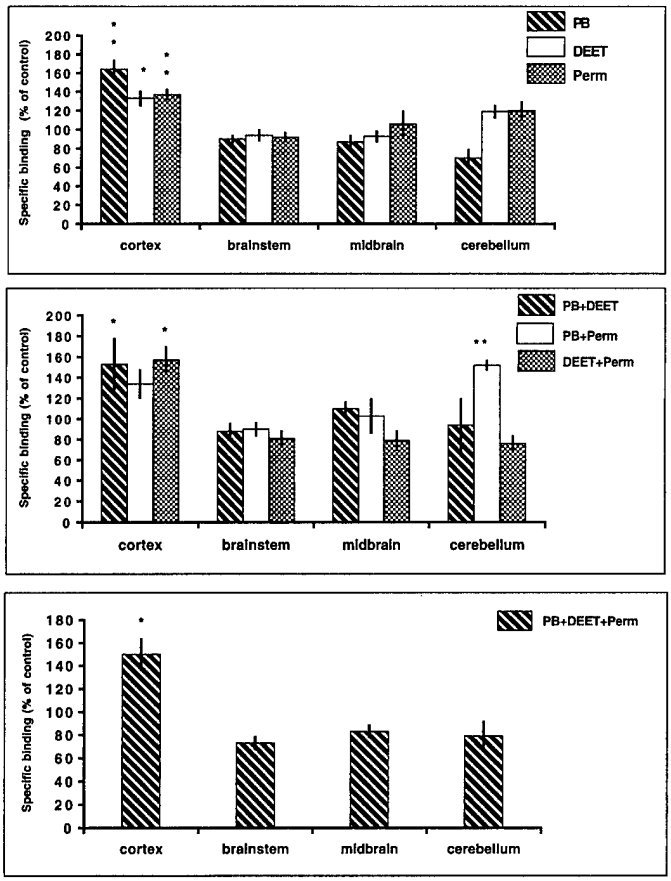

FIG. 5. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on m2 muscarinic acetylcholine receptor ligand binding in brain regions. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. Treatment with PB was given on the last 15 days of the experiment. The details of the treatment, membrane preparation, and [^{3}H]AFDX384 binding assay are elaborated in Materials and Methods. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. Data are presented as means ± SE (percent of control). p value: * < 0.01, ** < 0.006.

DISCUSSION

The present study examined effects of exposure to physiologically relevant doses of PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal), alone and in combination, on sensorimotor behavior and cholinergic system. The results suggest that exposure to these chemicals, alone or in combination, causes significant sensorimotor deficit. Furthermore, these data also suggest that treatment with PB, DEET, and permethrin, alone and in combination, causes differential regulation of AChE and m2 muscarinic and nicotinic acetylcholine receptors in the CNS.

The anatomical and molecular bases of the behavioral effects observed in the present study are complex. Different lesion studies have shown that severe sensorimotor impairment occurs in the animals with lesions of anteromedial and caudal forelimb cortex (Barth *et al.*, 1990). Similarly, studies with bilateral large lesions in the rat somatic sensorimotor cortex have shown impairment in limb-placing response. Additionally, it has also been suggested that limb placing is a function of corticospinal tract (Hicks and D'Amato, 1975). Thus, it is possible that treatment with PB, DEET, and permethrin, alone or in combination, could affect these innervations as well as innervations in other brain regions, and as a consequence, sensorimotor deficit may occur after prolonged exposure.

Beam-walking performance is an integrated form of behavior necessitating pertinent levels of consciousness, memory, sensorimotor, and cortical functions mediated by cortical area, and it has been suggested that an injury to cortex is reflected by a deficit in beam-walk task. A role for norepinephrine (NE) has strongly been proposed in the deficit caused by cortical injury (Boyeson *et al.*, 1992; Goldstein, 1995). It has been suggested

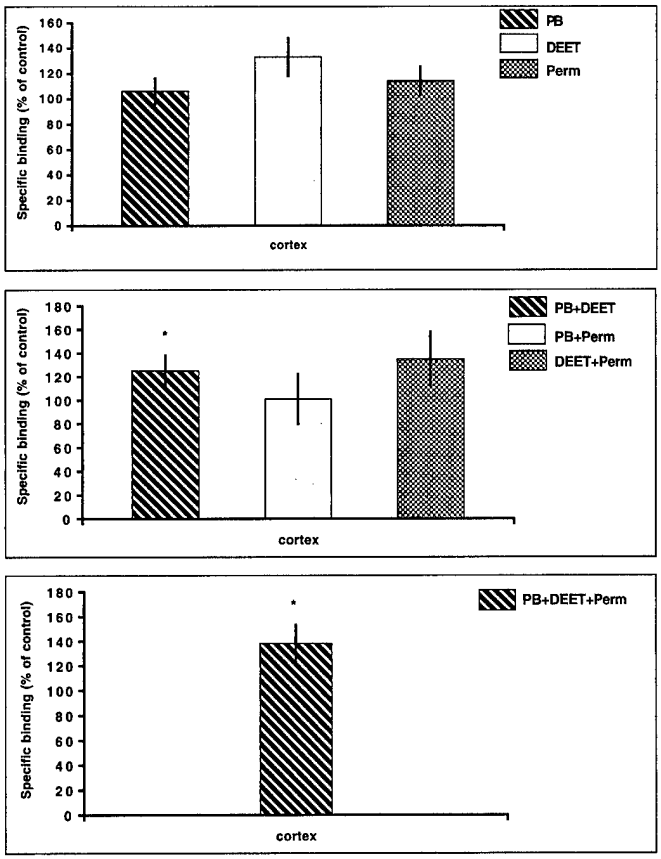

FIG. 6. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on nicotinic acetylcholine receptor ligand binding in cortex. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. Treatment with PB was given on the last 15 days of the experiment. The details of the treatment, membrane preparation, and [^{3}H]cytisine binding assay are elaborated in Materials and Methods. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. Data are presented as means ± SE (percent of control). p value: * < 0.03.

that NE facilitates the recovery from locomotor deficit by alleviating injury-induced decrease and turnover of NE levels in the cortex (Boyeson and Feeney, 1990; Kikuchi *et al.*, 2000). NE originates from the locus coeruleus and is widely distributed in the CNS, including cerebral cortex, hippocampus, cerebellum, and spinal cord. Therefore, it is possible that treatment with PB, DEET, and permethrin, alone and in combination, might regulate the noradrenergic and other catecholaminergic pathway in these animals. However, in view of the complexity of the behavioral outcome, it is also possible that these deficits are the result of exposure of these chemicals on multiple regions in the brain

PB has been used to protect against organophosphate nerve agent poisoning. It provides protection by shielding the peripheral AChE by reversibly binding to it. Because PB is a positively charged quaternary ion, it does not cross the BBB under ordinary circumstances. Thus, the toxic effects of PB are thought to be mediated through peripheral ACh nicotinic and muscarinic receptors (Albuquerque *et al.*, 1997). Indeed, Chaney *et al.* (1999) found that PB-induced seizures in the mouse were mediated via peripheral nervous system (PNS) muscarinic and nicotinic receptors. However, other studies also suggest that PB toxicity is mediated through CNS ACh receptors as well as through the PNS (Servatius *et al.*, 1998). Our results indicate that low-dose PB treatment for 15 days inhibited midbrain AChE activity, whereas plasma BChE activity showed little inhibition. It is possible that PB entry into the CNS and the consequent inhibition of AChE in the CNS may enhance the toxic potential of neurotoxic agents. It is not certain yet how PB could affect the CNS. It is possible that continuous treatment PB, alone or in combination with DEET and permethrin, could affect the BBB permeability, thus allowing PB to enter the CNS. Other studies also demonstrate that treatment with chemicals that cause inhibition of AChE lead to m2 mAChR up-regulation (Majocha and Baldessarini, 1984; Witt-Enderby *et al.*, 1995).

Some PGW veterans were exposed to a combination of pesticides and insecticides such as DEET and permethrin. In addition, they were exposed to PB because they were allowed to ingest twenty-one 30-mg tablets of PB. DEET is highly permeable to the skin and has been studied (Baynes *et al.*, 1997; Selim *et al.*, 1995) for its metabolism and toxicity. Permethrin has been used to impregnate the clothing of military personnel as protection against pestiferous and vector insects (Taplin and Meinking, 1990). It is possible that combined exposure to these chemicals would result in differential effects than exposure to single chemicals. Indeed our biochemical data show this phenomenon. Our data on cholinesterase suggest that DEET and permethrin alone do not inhibit the AChE activity in the CNS or plasma, whereas a combination with PB resulted in significant inhibition in brainstem and midbrain activity. These data are consistent with our previous studies in chickens (Abou-Donia *et al.*, 1996). An intriguing finding in our study is that treatment with permethrin alone caused a significant increase in cortical and cerebellar AChE activity, whereas DEET treatment alone caused a significant increase in brainstem AChE activity. The combination treatment led to a significant inhibition of AChE activity in brainstem, suggesting that brainstem may be the most susceptible to combined exposure. This inhibition may be mediated by PB, which might gain entry in the CNS following coexposure with DEET and permethrin. Treatment with DEET or permethrin caused an increase in AChE activity that may be due to an increase in AChE protein levels. Although not universally accepted, an increase in AChE protein may reflect an increased axonal repair and synaptic modeling, as has been shown recently (Bigbee *et al.*, 2000; Guizzetti *et al.*, 1996; Sternfeld *et al.*, 1998). Therefore, it is possible DEET and permethrin treatment alone may cause subtle changes that are reflected in increased synaptic modeling and repair. The behavioral data on single chemicals substantiate this notion. Coexposure with PB, DEET, and permethrin together caused a significant inhibition in brainstem and midbrain AChE activity, suggesting that treatment with three chemicals together could lead to added neurotoxic effects.

Cholinergic system in the CNS plays an important role in learning and memory (Lena and Changeux, 1998; Levey *et al.*, 1995). We studied the receptor ligand binding for m2AChR and nicotinic AChR in the cortex. Based on our data, it appears that increased receptor ligand binding density for both of the receptors in the cortex in response to treatment with PB, DEET, and permethrin, alone or in combination, may be a compensatory mechanism for a reduced ability of these receptors to bind their respective ligands. It is known that treatment with muscarinic antagonists induces receptor up-regulation (Ben-Barak and Dudai, 1980; Coccini *et al.*, 2000; Majocha and Baldessarini, 1984; Smiley *et al.*, 1998). Wang *et al.* (1996) reported the regulation of muscarinic receptor by repeated treatment with nicotine. The up-regulation of cortex m2AChR may be related to an increase in the AChE levels in the cortex of the animals treated with DEET or permethrin. Increased AChE activity in the cortex suggests that ACh levels are depleted. It is possible that subsequent receptor up-regulation is a response to reduced neurotransmitter levels. Increased ligand binding for m2 muscarinic receptor results in the inhibition of adenylate cyclase activity through a pertussis toxin-sensitive G-protein, resulting in an inhibitory postsynaptic response (Brann *et al.*, 1993; Wess, 1996). The inhibitory nature of m2 receptor may have regulatory response on (GABA)ergic system in the cortex. It is known that cholinergic input in certain brain regions tonically inhibits (GABA)ergic system and is inhibitory to vasomotor glutamergic neurons. Thus, an increase in m2AChR in response to treatments with PB, DEET, and permethrin, alone or in combination, may regulate the glutamergic pathway leading to a decreased motor response. Also, it is well accepted that most of the toxic effects of pyrethroid insecticides are mediated through the modification of axonal Na^+ channels (Narahashi, 1996). Moreover, there is

additional evidence that some of the toxic effects of pyrethroids are mediated by the interaction with GABA receptor-ionophore complex (Crofton and Reiter, 1987; Gammon and Sander, 1985; Lawrence *et al.*, 1985). However, no clear association between the modification of Na^+ channels and development of sensorimotor deficit has yet been established.

In summary, our results suggest that exposure to physiologically relevant doses of PB, DEET, and permethrin, alone or in combination, lead to sensorimotor deficits and alteration in the cholinergic system in rats. These results further suggest that exposure with these chemicals, alone or in combination, may have played a role in the development of long-term health consequences associated with the PGW veterans. The contribution of cholinergic changes to the behavioral deficit following treatment with these chemicals is not clear at the moment, as these changes may involve a combination of mechanisms related to central and peripheral or neuromuscular system. In a recent study, Nostrandt *et al.*, (1997) observed an insignificant correlation between changes in muscarinic receptor and AChE in the CNS following treatment with chlorpyrifos, which is a more potent cholinotoxic than the chemicals we used in the current studies. However, the possibility remains that the behavioral impairment observed in our studies may also have been a consequence of other generalized abnormalities such as deficit in cognition and motivation because of the changes in cholinergic system. Further studies are in progress to evaluate the histopathological correlates of these behavioral changes.

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Appendix 7
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EFFECTS OF DAILY DERMAL APPLICATION OF DEET AND PERMETHRIN, ALONE AND IN COMBINATION, ON SENSORIMOTOR PERFORMANCE, BLOOD–BRAIN BARRIER, AND BLOOD–TESTIS BARRIER IN RATS

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DEET and permethrin were implicated in the development of illnesses in some veterans of the Persian Gulf War. This study was designed to investigate the effects of daily dermal application of these chemicals, alone or in combination, on the permeability of the blood–brain barrier (BBB) and blood–testes barrier (BTB) and on sensorimotor performance in male Sprague-Dawley rats. Groups of five rats were treated with a dermal daily dose of 4, 40, or 400 mg/kg DEET in ethanol or 0.013, 0.13, or 1.3 mg/kg permethrin in ethanol for 60 d. A group of 10 rats received a daily dermal dose of ethanol and served as controls. BBB permeability was assessed by injection of an iv dose of the quaternary ammonium compound [^{3}H]hexamethonium iodide. While permethrin produced no effect on BBB permeability, DEET alone caused a decrease in BBB permeability in brainstem. A combination of DEET and permethrin significantly decreased the BBB permeability in the cortex. BTB permeability was decreased by treatment with DEET alone and in combination with permethrin. The same animals

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underwent a battery of functional behavior tests 30, 45, and 60 d after exposure to evaluate their sensorimotor abilities. All treatments caused a significant decline in sensorimotor performance in a dose- and time-dependent manner. These results show that daily dermal exposure to DEET, alone or in combination with permethrin, decreased BBB permeability in certain brain regions, and impaired sensorimotor performance.

The insect repellent *N,N*-diethyl-*m*-toluamide (DEET) and the insecticide pyrethroid permethrin [3-phenoxybenzyl-(±)-*cis,trans*3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate have been used extensively by humans since their introduction. DEET is commonly used as an effective repellent against mosquitoes, flies, ticks, and other insects in the form of lotion, stick, or spray (Robbins & Cherniack, 1986; McConnell et al., 1986). Extensive and repeated topical application of DEET can result in human and animal poisoning including death (Gryboski et al., 1961; Roland et al., 1985; Edwards & Johnson, 1987; McCain et al., 1997). The symptoms associated with DEET poisoning are characterized by tremors, restlessness, difficulty with speech, seizures, impairment of cognitive function, and coma (McConnell et al., 1986). High levels of DEET exposure have been reported to cause spongiform myelinopathy (Verschoyle & Aldridge, 1990). Because DEET efficiently crosses the dermal barrier (Windheuser et al., 1982; Hussain & Ritschel, 1988; Stinecipher & Shah, 1997) and localizes in dermal fat deposits (Blomquist & Thorsell, 1977; Snodgrass et al., 1982), it is possible that DEET could enhance the availability of drugs and toxicants to other organs, including the brain (Stinecipher & Shah, 1997).

Permethrin is a type I synthetic pyrethroid insecticide that exists in four different stereoisomers (Casida et al., 1983). It provides insecticidal activity for several weeks following a single application. Permethrin toxicity is due to prolonged opening of the sodium channels, leading to repetitive discharges after a single stimulus (Narahashi, 1985). This repetitive nerve action is associated with tremors, hyperactivity, ataxia, convulsions, and, in some cases, paralysis.

During the Persian Gulf War, some service personnel were exposed to a variety of chemicals, including DEET and permethrin (Institute of Medicine, 1995; Abou-Donia et al., 1996). The reported exposure to DEET and permethrin for Gulf War veterans could have occurred during their deployment because these chemicals were used as protective insecticide. Some veterans have reported chronic symptoms including headache, loss of memory, fatigue, muscle and joint pain, and ataxia. All of these symptoms involve either central or peripheral nervous systems function. In these experiments, we studied the effects of DEET and permethrin alone or in combination on blood–brain barrier (BBB) and blood–testes barrier (BTB) permeability and sensorimotor functions following daily dermal application.

MATERIALS AND METHODS

Chemicals

Technical-grade (93.6%) permethrin (±)-*cis/trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester was obtained from Roussel Uelaf Corp., Pasadena, TX. DEET (97.7%, *N,N*-diethyl-*m*-toluamide), acetylthiocholine iodide, and butyrylthiocholine iodide were purchased from Sigma Chemical Co., St. Louis, MO. [^{3}H]-Hexamethonium iodide, specific activity 18 Ci/mmol, was obtained from USAMRID, Aberdeen Proving Ground, MD. All other chemicals and reagents were of the highest purity available from commercial sources.

Animals

Male Sprague-Dawley rats (200–250 g) obtained from Zivic Miller, Allison Park, PA, were used. Animals were randomly assigned to control and treatment groups and housed at 21–23°C with a 12-h light/dark cycle. They were supplied with Purina certified rodent chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. The rats were allowed to adjust to their environment for a week before treatment. Animal care was in accordance with institutional guidelines.

Treatment

For dermal application of the chemicals, 1 in^2 of the back of the neck was shaved. The chemicals were applied on the shaved area to give the desired concentration of test compounds in 0.2 ml vehicle. Groups of 10 rats received a daily topical dose of 4, 40, or 400 mg/kg DEET (0.1×, 1×, and 10× dose) in 70% ethanol or 0.013, 0.13, or 1.3 mg/kg (0.1×, 1×, and 10× dose) permethrin in 70% ethanol, alone or in combination. Control animals received an equal volume of the vehicle. The treatment was carried out daily, 7 d/wk, for 60 d. The 1× dose of DEET and that of permethrin are based on an estimate of exposure that may have occurred to army personnel during Gulf War. For combined exposure, each chemical was given at 0.1×, 1×, or 10× concentration.

For BBB studies, 24 h after the last treatment, subgroups of 5 animals were anesthetized and then injected in the tail vein under anesthesia with [^{3}H]hexamethonium iodide (10 μCi, mixed with cold hexamethonium iodide) to give a final dose of 0.7 mg/kg (1 μCi/kg). After 10 min, animals were anesthetized with ketamine/xylazine, blood was collected from the heart with heparinized syringes, and the animals were sacrificed by decapitation. Brains and testes were removed and placed in ice-cold normal saline. Brain regions were dissected on ice into cortex, brainstem, midbrain, and cerebellum. Following dissection, the brain regions and testes were snap frozen in liquid nitrogen. Plasma was separated from whole blood by centrifugation. Plasma, brain regions, and testes were stored at –20°C for later analysis.

For the determination of [^{3}H]hexamethonium iodide uptake in tissues and plasma, a weighed amount was subjected to oxygen combustion using a Packard 306B tissue oxidizer (Packard Instrument Co., Downers Grove, IL). Total radioactivity present in tissues and plasma was determined in triplicate in a Beckman LS-6500 multipurpose scintillation spectrometer (Beckman Instruments Corp., Palo Alto, CA). The radioactivity was calculated as disintegrations per minute (dpm) per gram tissue divided by dpm per milliliter plasma for each animal.

Statistical Analysis

The data from BBB experiments were analyzed by analysis of variance (ANOVA) for the test of significance. The criterion for significance was set at $p < .05$. The graphs were generated on Excel graphics for Macintosh and are presented as mean ± SE of percent of control.

Behavioral Studies

A battery of standardized tests was employed. These behavioral tests were designed to measure sensorimotor reflexes, motor strength, and coordinated gait (Bederson et al., 1986; Markgraf et al., 1992; Goldstein, 1993). All behavioral testing was performed by trained observers blind to the animals' treatment status and was carried out in a soundproof room with subdued lighting (less than 10.76 lumen/m^2, ambient light). Rats were handled for 2 min daily for 5 d during the week prior to behavioral testing.

Postural Reflexes

Description Rats are held gently by the tail, 1 m above the floor, and were observed for forelimb extension. Normal rats extend both forelimbs. Consistent flexion of the forelimb is an abnormal response. Rats with consistent forelimb flexion are then further assessed by placing each on a large sheet of plastic-coated paper that can be gripped with the forepaws. With the tail held, gentle lateral pressure is applied behind the rat's shoulder until the forelimb slides several inches. The maneuver is repeated five times in each direction. Normal rats resist lateral pressure by gripping the coated paper.

Scoring Grade 0 was given to rats without evidence of consistent forelimb flexion when held above the floor; grade 1, to rats with consistent forelimb flexion; and grade 2, to otherwise grade 1 rats that do not resist lateral pressure on at least three of five trials in either direction.

Limb Placing

Description Visual, tactile, and proprioceptive forelimb placing responses were examined. For visual placing, rats are held in the examiners' hands 10 cm above the tabletop with forelimbs hanging free. The rats are then slowly tilted toward the table. Intact rats reach toward the table with both forepaws. For tactile placing, the dorsal and then lateral portions of

the forepaws are touched to the table edge. Intact rats immediately place the paw on the surface of the table. Proprioceptive placing is tested by pushing the forepaw onto the table edge. Care is taken to avoid the vibrissae touching the table.

Scoring For each test, grade 0 is assigned if the placing response is immediate; grade 1, if the placing response is slow or delayed; and grade 2, if the placing response does not occur within 2 s.

Orienting to Vibrissae Touch

Description The rat is placed atop an inverted polycarbonate cage and allowed 1 min for habituation. Its vibrissae are then touched with a cotton tipped swab.

Scoring Grade 0 is assigned if the rat orients to the side of the probe on at least two of three trials from each side, and grade 1 if the rat fails to orient on at least two of three trials on either side.

Grip Time

Rats' forepaw grip time was assessed by having them hang from a 5-mm-diameter wood dowel gripped with both forepaws. Time to release their grip was recorded in seconds.

Beam Walking and Beam Score

Description The testing apparatus is a 2.5 × 122-cm wooden beam elevated 75.5-cm above the floor with wooden supports. A 20 × 25 × 24-cm goal box with a 9.5-cm opening is located at one end of the beam. A switch-activated source of bright light (75-W tungsten bulb) and a source of white noise (41 dB at 8000 Hz, 58 dB at 4000 Hz, 56 dB at 2000 Hz, 56 dB at 1000 Hz, 58 dB at 500 Hz, and 52 dB at 250 Hz SPL at the center of the frequency at each octave band) were located at the start end of the beam and served as avoidance stimuli. The rats are first trained to traverse the beam with a series of three approximate trials (i.e., rats are first placed at the entrance to the goal box, then at the mid portion of the beam, and finally at the start end of the beam). Rats are readily trained to perform the beam-walking task (Goldstein, 1993). For the testing trials, the rat was placed at the start end of the beam, near the sources of light and noise.

Scoring Beam-walking ability is measured with a seven-point scoring system scale as previously described (Goldstein, 1993): 1, the rat is unable to place the affected hind paw on the horizontal surface of the beam; 2, the rat places the affected hind paw on the horizontal surface of the beam and maintains balance for at least 5 s; 3, the rat traverses the beam while dragging the affected hind paw; 4, the rat traverses the beam and at least once places the affected hind paw on the horizontal surface of the beam; 5, the rat crosses the beam and places the affected hind limb on the horizontal surface of the beam to aid less than half its steps; 6, the

rat uses the affected hindpaw to aid more than half its steps; and 7, the rat traverses the beam with no more than two footslips. In addition, the latency until the animal's nose enters the goal box (up to 90 s) is recorded for the final trial. Rats that fall off the beam are assigned latencies of 90 s.

Inclined Plane

The rats were placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised, as described by Yonemori et al. (1998). The board was slowly rotated to the vertical position. Two trials were performed for each testing session.

Scoring The angle at which the rat begins to slip downward was recorded. The results of two trials were averaged at each time point.

Statistical Analysis

For continuous data, groups were compared by two-way repeated-measures ANOVA. The significance of post hoc pairwise comparison was determined with Fisher's least significant difference (LSD) tests. If indicated for nonparametric data, comparisons across treatment groups were made with the Kruskal–Wallis test.

RESULTS

General Observations

The clinical condition of animals treated with daily dermal application of 4, 40, or 400 mg/kg DEET in ethanol and 0.013, 0.13, or 1.3 mg/kg permethrin in ethanol alone or in combination was not different from controls. No difference was also observed in the weights of treated animals as compared with control.

Blood–Brain Barrier and Blood–Testis Barrier Permeability

Effects of 60 daily dermal applications of DEET and permethrin, alone and in combination, at 3 dose levels (0.1×, 1×, and 10×), on BBB integrity are presented in Figures 1–3 and are given as percent of control of the mean of the ratio between brain and plasma [^{3}H]hexamethonium iodide uptake. A decreased uptake of [^{3}H]hexamethonium iodide was observed in the brainstem of the animals treated with DEET at 1× and 10× dose when compared with the control group (Figure 1). Brainstem was the most affected by DEET treatment, with a decrease of 78, 66, and 65% observed at 0.1×, 1×, and 10× doses, respectively. DEET treatment alone also caused a decrease in BTB permeability. Animals treated with permethrin at all the three doses did not demonstrate any significant changes in BBB permeability in the brain or in the testes when compared to control animals (Figure 2). However, animals treated with DEET + permethrin did exhibit a decrease in BBB permeability (Figure 3). The decrease in BBB permeability in the cortex was exacerbated by the combination of DEET

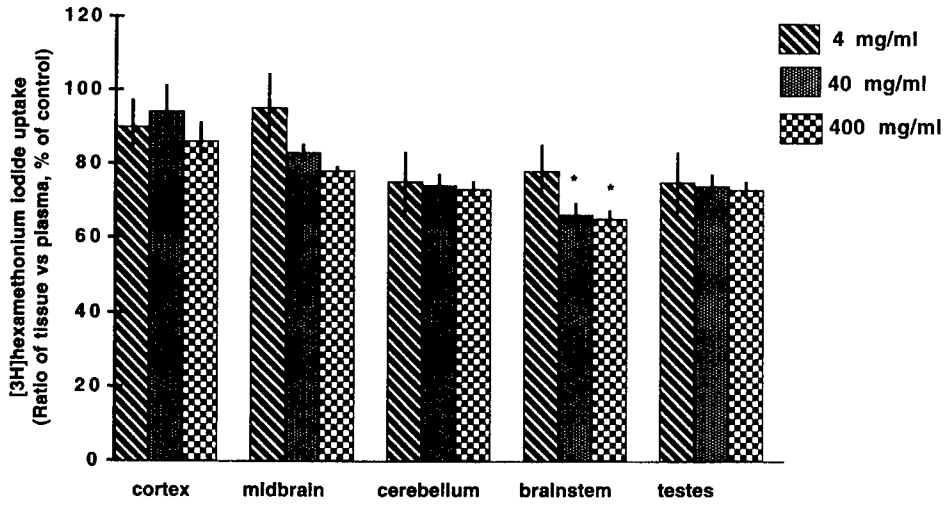

FIGURE 1. [^{3}H]Hexamethionium iodide uptake in brain regions and testes of the animals treated with DEET. Animals were treated with 60 daily doses of 4 mg/kg, 40 mg/kg, or 400 mg/kg DEET (0.1×, 1×, and 10× dose) by dermal application. [^{3}H]Hexamethonium iodide was given iv and brain regions were dissected. Radioactivity was measured in each region as described in Materials and Methods. The values represent the ratio between tissue uptake and plasma and are presented as mean ± SE of percent of control. The control values were: cortex, 0.32 ± 0.05; midbrain, 0.48 ± 0.06; cerebellum, 0.47 ± 0.09; brainstem, 0.45 ± 0.10; testes, 0.47 ± 0.09. Asterisk indicates p value < .05.

with permethrin ($p < .02$ at 1× and $p < .01$ at 10× dose). These animals showed a significant decrease in BBB permeability in the cortex that ranged from 86% to 72% of control.

Behavioral Performance

Each compound was given alone and in combination ($n = 5$ per group) at 1 of 3 doses (0.1×, 1×, and 10×) with a series of behavioral testing performed on d 30, 45, and 60. Figure 4 gives beam walking scores; Figure 5 beam walking times; Figure 6, inclined plane responses; and Figure 7 forepaw grip time for each treatment and for each time point. There was no effect of any of the drugs on postural reflexes, limb placing, or vibrissae touch (data not shown).

For beam walk score (Figure 4), control animals consistently showed completely normal performance aside from the 30-d testing in the permethrin experiment. The graphs otherwise show a dose response with greatest deficits at increasing dose as well as evidence of progressively declining performance over time for animals that received permethrin alone or a combination of DEET and permethrin. For DEET, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 17.3$, $p < .0001$) and a significant dose × time interaction (ANOVA $F_{3,6} = 9.7$, $p < .0001$). For the combination, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,13} = 267.6$, $p < .0001$) and a signifi-

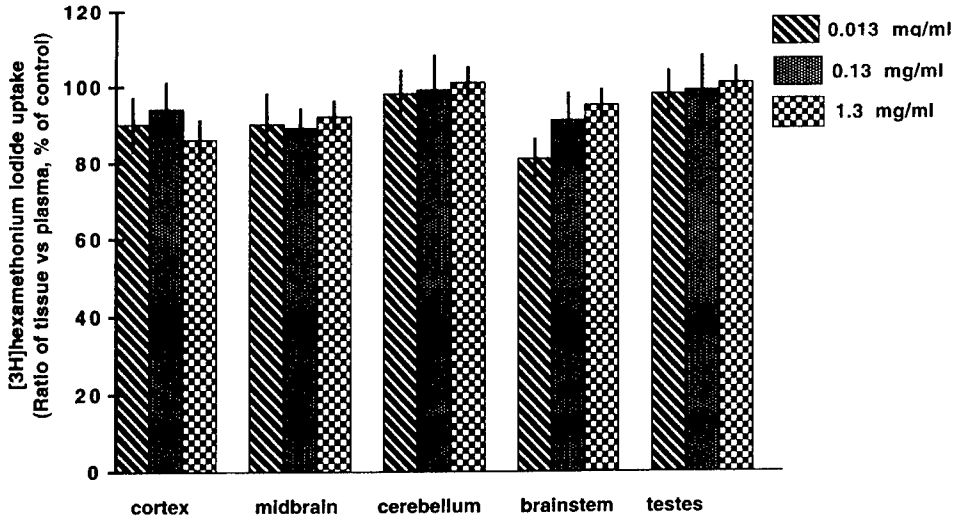

FIGURE 2. [^{3}H]Hexamethionium iodide uptake in brain regions and testes of the animals treated with permethrin. Animals were treated with 60 daily doses of 0.013 mg/kg, 0.13 mg/kg, or 1.3 mg/kg permethrin (0.1×, 1×, and 10× dose) by dermal application. [^{3}H]Hexamethionium iodide was given iv, and brain region and testes were dissected. All other details were as described for Figure 1. The values represent the ratio between tissue uptake and plasma and are presented as mean ± SE of percent of control. The control values were: cortex, 0.26 ± 0.05; midbrain, 0.28 ± 0.03; cerebellum, 0.24 ± 0.03; brainstem, 0.25 ± 0.04; testes, 0.24 ± 0.02.

cant dose × time interaction (ANOVA $F_{3,6} = 3.9$, $p = .0047$). At the 30-d time point, the 0.1× dose of DEET ($p < .0001$) but neither permethrin nor the combination differed from controls.

For beam walk time (Figure 5), there were also significant relationships between each compound and performance. For DEET, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 144.3$, $p < .0001$) but no dose × time interaction (ANOVA $F_{3,.6} = 1.94$, $p = .2457$). For permethrin, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 1.78$, $p = .0001$) and a significant dose × time interaction (ANOVA $F_{3,6} = 3.74$, $p < .0062$). For the combination, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 86.0$, $p < .0001$) and a significant dose × time interaction (ANOVA $F_{3,6} = 10.2$, $p = .0047$). At the 30-d time point, the 0.1× dose of DEET ($p < .0001$) but neither permethrin nor the combination differed from controls.

Inclined plane performance tests (Figure 6) showed significant relationships between each compound and performance. For DEET, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,6} = 4.21$, $p < .001$). For permethrin, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 26.6$, $p < .0001$) and a significant dose × time interaction (ANOVA $F_{3,6} = 3.74$, $p = .0042$). For the combination, two-way repeated measures ANOVA showed a signifi-

cant effect of dose (ANOVA $F_{3,16} = 24.9$, $p < .0001$) and a significant dose × time interaction (ANOVA $F_{3,6} = 10.2$, $p = .0035$). At the 30-d time point, the 0.1× dose of DEET ($p = .04$), and the combination ($p = .005$) differed from controls. Permethrin did not differ from controls significantly ($p = .06$).

Finally, for forepaw grip test (Figure 7), there was a clear effect of all doses at all time points. For DEET, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 18.7$, $p = .0060$). For permethrin, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 82.1$, $p < .0001$). Because all groups were severely affected even at the first time point, the dose × time interactions were not significant. At the 30-d time point the 0.1× dose of DEET, permethrin, and the combination each differed significantly from controls ($p < .001$, respectively).

The relationships between each test compound and dose in comparison to pooled controls are shown in Figure 8 for the 30-d time point. Declining performance with increasing dose on beam walking is apparent for animals that received DEET or the DEET–permethrin combination (top panels); poorer inclined plane responses with increasing dose are present for animals that received permethrin or the DEET–permethrin combination (bottom left panel); and all animals at all doses had impaired paw grip strength (bottom right panel).

A comparison of the significance at the lowest and highest dose of each agent compared to the combination at the 30-d time point for

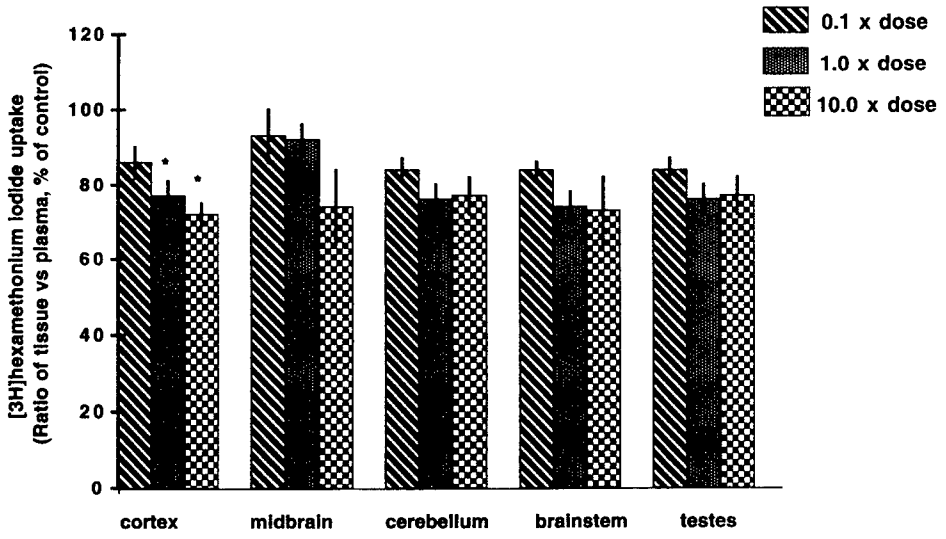

FIGURE 3. [^{3}H]Hexamethonium iodide uptake in brain region and testes of the animals treated with DEET and permethrin together by dermal application for 60 d. All other details were as described for Figure 1. The values represent the ratio between tissue uptake and plasma and are presented as mean ± SE of percent of control. The control values were: cortex, 0.41 ± 0.03; midbrain, 0.49 ± 0.03; cerebellum, 0.42 ± 0.02; brainstem, 0.40 ± 0.03; testes, 0.42 ± 0.03. Asterisk indicates p value < .05.

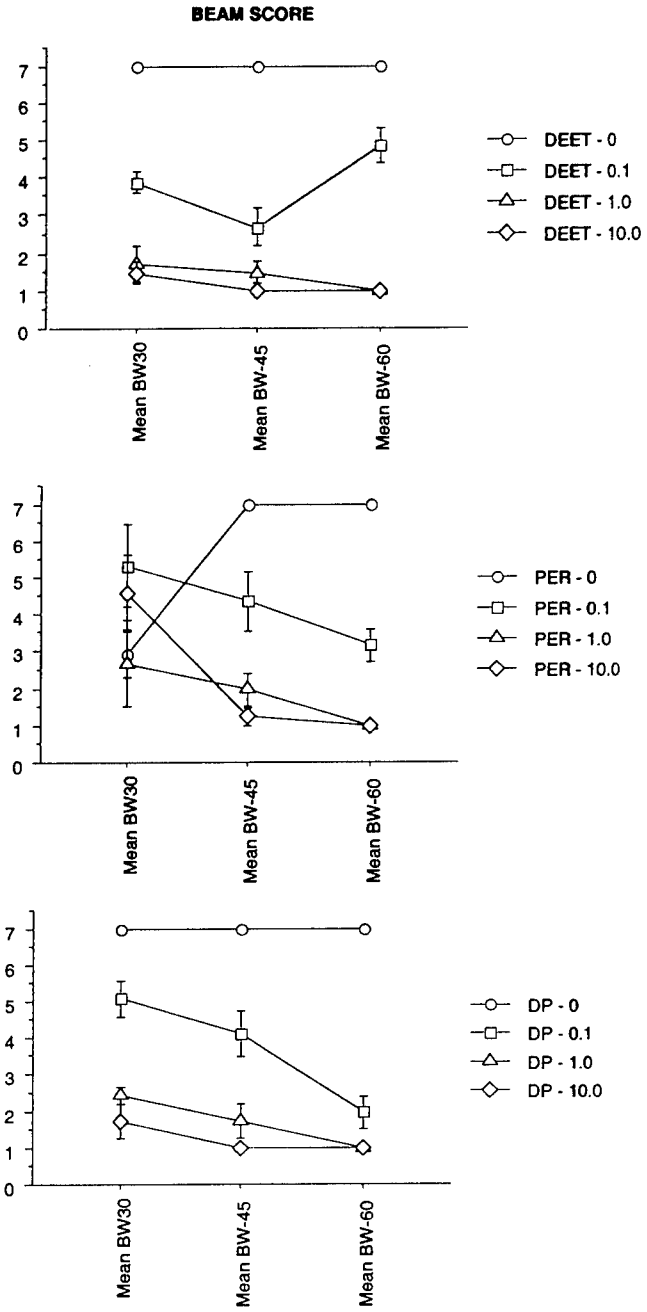

FIGURE 4. Beam score response of animals treated with DEET or permethrin or a combination of DEET and permethrin for 60 d by dermal application. The doses are represented as 0.1×, 1×, or 10× of real-life exposure, which is equivalent to 4, 40, and 400 mg/kg for DEET and 0.013, 0.13, and 1.3 mg/kg of permethrin, respectively. The animals were examined on d 30, 45, and 60, as presented on the *x* axis. The *y* axis presents the mean of the beam score. Evaluations were carried out as described in Materials and Methods.

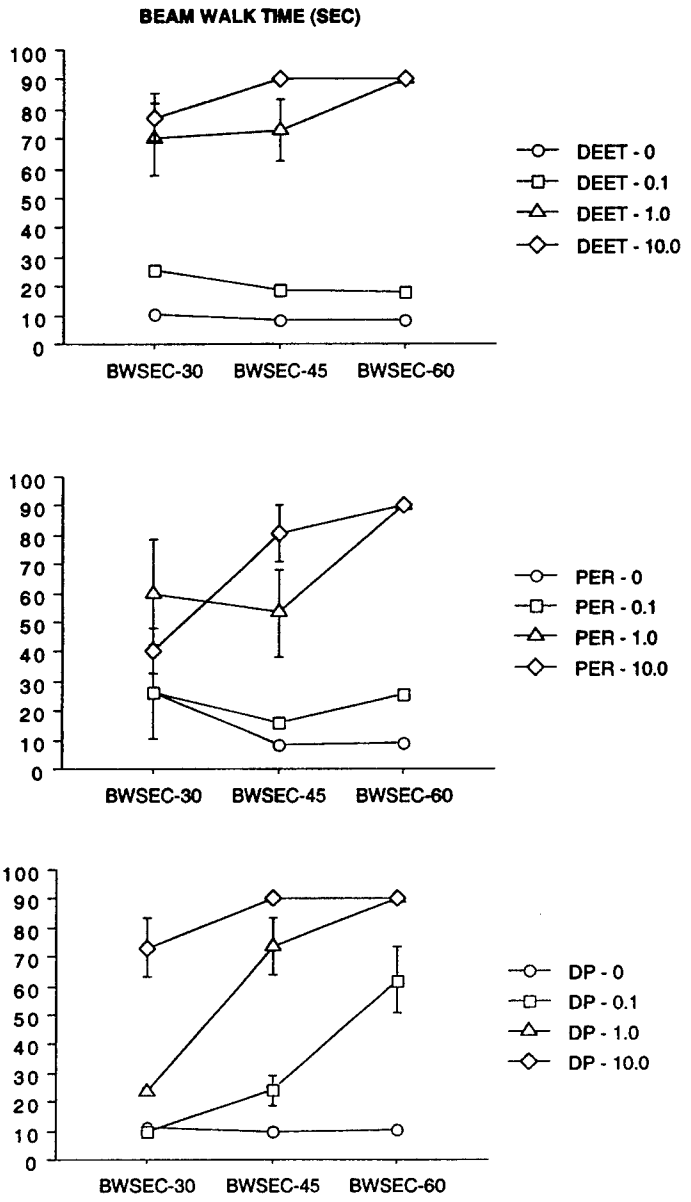

FIGURE 5. Beam walk time in seconds of the animals treated with DEET or permethrin or a combination of DEET and permethrin for 60 d by dermal application. All the other details were as described for Figure 4.

each behavioral parameter is presented in Table 1. There were significant differences between the highest dose in combination and permethrin alone in beam walk score and beam walk time, and between the highest dose of the combination and DEET for inclined plane performance.

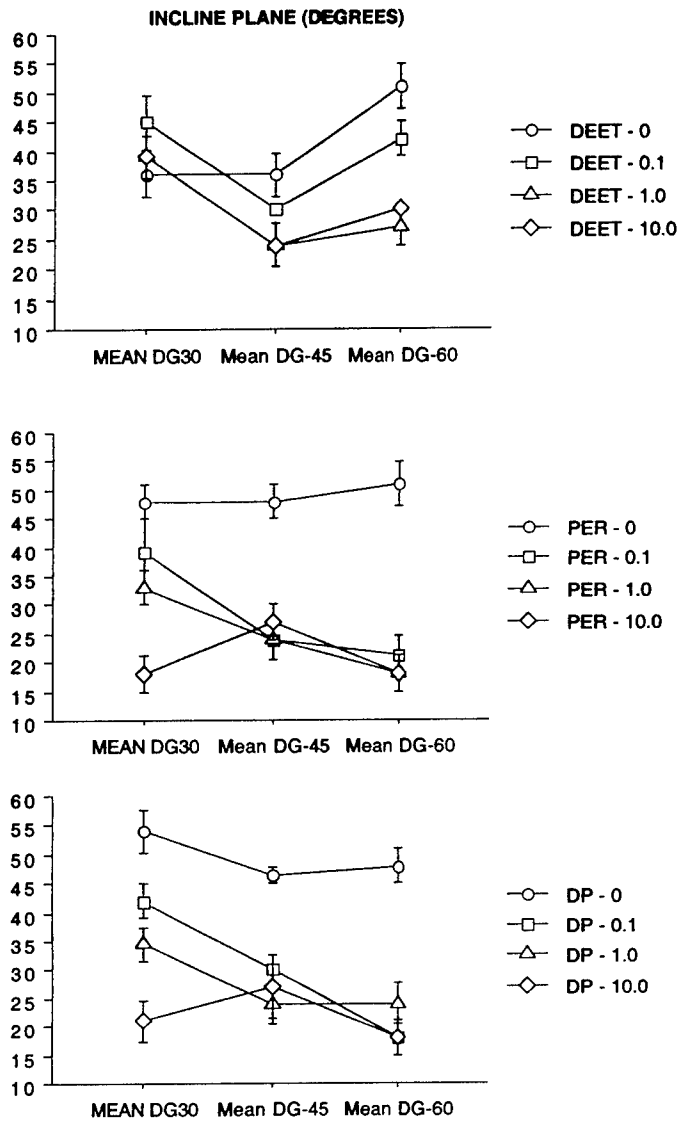

FIGURE 6. Incline plane in degrees of the animals treated with DEET or permethrin or a combination of DEET and permethrin together for 60 d by dermal application. The data on the *y* axis represents the mean of the angles in degrees at which animal falls off the plane as described. All other details were as described for Figure 4.

In summary, these studies demonstrate impaired behavioral performances at even the lowest administered dose of each agent for at least some sensorimotor parameters. The combination of the two agents resulted in significantly poorer performance than either agent alone, but only for some behavioral parameters and only at high dose.

DISCUSSION

This study was designed to investigate the effects of repeated daily dermal application of DEET and permethrin alone or in combination on sensorimotor function and the integrity of the BBB in male Sprague-Dawley rats. The route of exposure and the dose levels of test compounds were

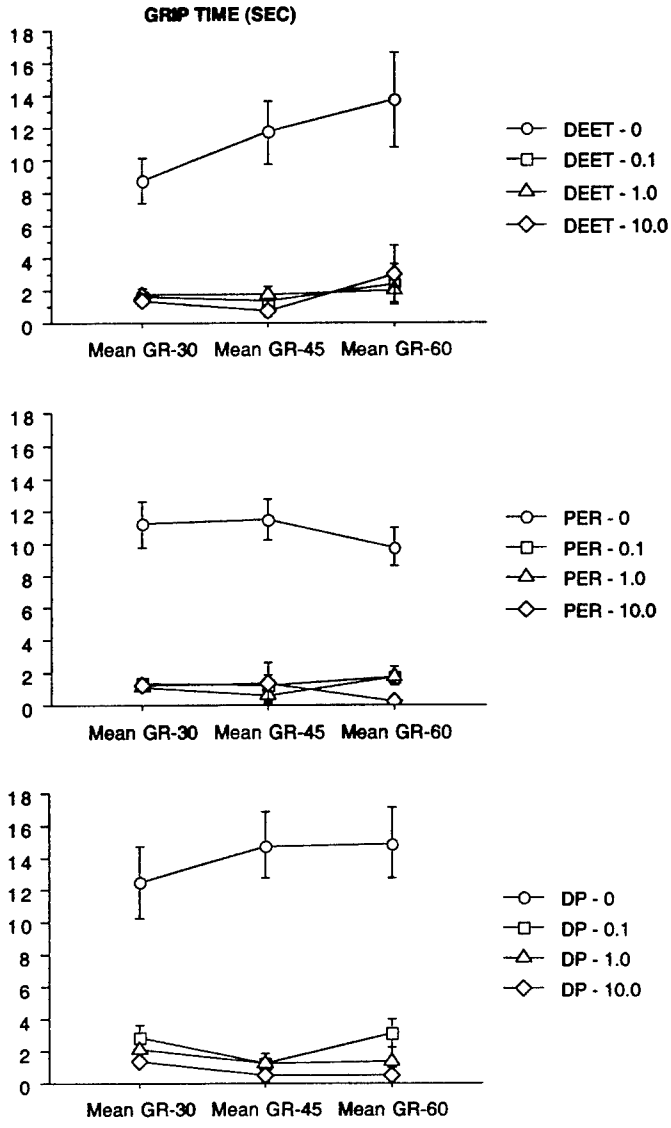

FIGURE 7. Grip time in seconds of the animals treated in the DEET or permethrin or DEET and permethrin in combination for 60 d by dermal application. Numbers on the *y* axis represents the time in seconds the animals are able to hang from a wooden dowel griped with both forepaws. The *x* axis represents the days when the evaluation was carried out.

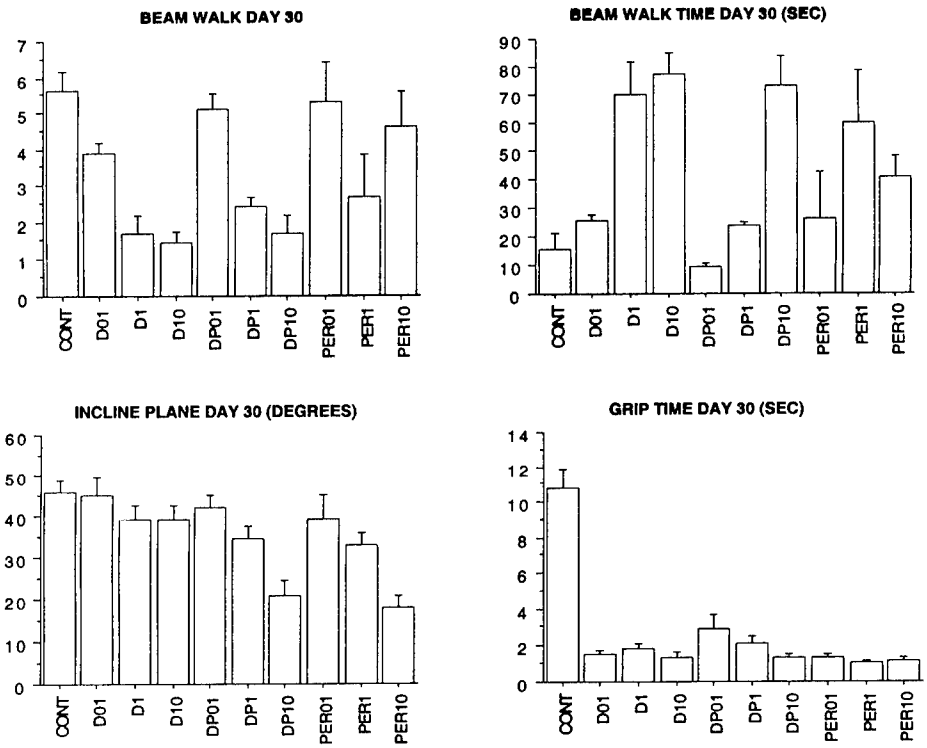

FIGURE 8. Presentation of the data as bar graph for the d 30 observation on the effects of DEET or permethrin or a combination of DEET and permethrin on behavioral performance.

chosen to closely reflect those present during the Gulf War. Both test compounds were applied dermally using 0.1, 1, and 10 times the estimated real life doses of 40 mg/kg/d for DEET and 0.13 mg/kg/d for permethrin. Our data suggest that physiologically relevant exposure to DEET for 60 d caused a decrease in BBB permeability in the brainstem at 1× and 10× doses. Permethrin exposure alone had no observable effect on the BBB permeability. However, a combination of the two chemicals caused a decrease in the BBB permeability in the cortex in a dose-dependent fashion as compared to DEET or permethrin alone, suggesting that the two chemicals together may have additive effects, but only in selected brain region(s) such as cortex. In our earlier studies in hens we reported that coexposure with pyridostigmine bromide, DEET, and permethrin resulted in a higher level of toxicity than each chemical alone (Abou-Donia et al., 1996). In those studies we used a subcutaneous route of exposure at relatively higher doses of each chemical. These results suggest that DEET exposure alone or in combination by dermal application can lead to BBB permeability changes in certain brain region(s) that can have important physiological/pharmacological consequences.

The BBB regulates the entry of molecules into the central nervous sys-

tem (CNS) based on the size, charge, hydrophobicity, and/or affinity of carriers. The selective nature of the BBB helps maintain the homeostasis of the CNS environment to ensure proper brain function (Joo, 1996). The BBB consists of the cerebral capillary endothelium, which contains tight junctions. These tight junctions form rows of extensive overlapping occlusions that block the intercellular route of solute entry into the CNS. Any changes in the basal permeability of the cerebral capillary endothelium can exacerbate a variety of pathological processes. Our finding that DEET exposure alone or in combination with permethrin caused a decrease in BBB permeability could restrict passage of important molecules that are required for normal homeostasis in the CNS. Our studies do not provide any data on the levels of DEET or permethrin in the CNS. A recent in vitro study (Baynes et al., 1997) suggested that in coexposure with DEET and permethrin, DEET antagonizes the absorption of permethrin in different model systems of cutaneous exposure. The decrease in BBB permeability with combined exposure does not necessarily reflect an antagonistic effect. It could also mean that both chemicals in combination cause changes in cerebrovascular endothelium, leading to a decrease in [^{3}H]hexamethonium iodide uptake. The decrease in BBB permeability could be mediated by one of several mechanisms; for example, it is known that membrane fluidity changes induced by ethanol cause a decrease in the passage of [^{3}H]vincristine into the CNS (Domer & Smith, 1988). A decrease in blood flow to the CNS could also cause a reduction in the availability of [^{3}H]hexamethonium iodide to the CNS.

TABLE 1. Comparison of Lowest and Highest Dose of Each Agent or Combination at 30 d

Test	Dose	Comparison	p^a
Beam-walk score	Low	Comb vs. Per	NS
	High		<.02
	Low	Comb vs. DEET	NS
	High		NS
Beam-walk time	Low	Comb vs. Per	NS
	High		<.03
	Low	Comb vs. DEET	NS
	High		NS
Incline plane	Low	Comb vs. Per	NS
	High		NS
	Low	Comb vs. DEET	NS
	High		<.004
Paw grip	Low	Comb vs. Per	NS
	High		NS
	Low	Comb vs. DEET	NS
	High		NS

[a]Fisher LSD.

Cerebrovascular endothelium is known to express multidrug transporting *p*-glycoprotein (*p*-gp) at BBB sites (Gottesman & Pastan, 1993). It has been proposed that *p*-gp localization at the BBB serves to protect the CNS by causing efflux of drugs and chemicals. Indeed, deletion of the *p*-gp gene has been shown to lead to massive localization of chemotherapeutic changes in the brain, suggesting that *p*-gp plays an important role in efflux of drugs and chemicals (Schinkel et al., 1994). Therefore, it is possible that under our experimental conditions, DEET alone or in combination with permethrin may regulate the expression of *p*-gp. The neurotoxic effects of DEET may be augmented both by its increased localization into the CNS because of its lipophilicity, and because of decrease in the transport of otherwise critical molecules. It has also been reported that elevated cAMP levels decrease the BBB permeability in rat pial vessels and frog peripheral capillaries (He & Curry, 1993). Increased cAMP levels have been shown to reduce BBB permeability induced by cerebral ischemia (Belayev et al., 1998). It is possible that DEET could modulate the levels of cAMP after prolonged exposure. Neurotoxic chemicals induce a hypothermic response, which is known to reduce BBB permeability (Gordon, 1993). Prolonged exposure to DEET may cause a hypothermic response that may be responsible for BBB permeability changes. Tight gap junctions at the BBB are mediated by adherens (Rubin & Staddon, 1999). These are a class of membrane proteins that are critical for maintenance and functioning of BBB. DEET access to the microvascular endothelium could regulate the expression of such junction proteins, resulting in diminished blood flow, thereby reducing the entry of [^{3}H]hexamethonium iodide molecules. Decrease in the BBB permeability by DEET alone or in combination with permethrin could result by any or all of the mechanisms already discussed. It should be noted, however, that the changes observed in BBB permeability are subtle; therefore, additional approaches such as histopathological evaluations may provide definitive proof of the changes in the CNS occurring as a consequence of DEET treatment alone or in combination with permethrin.

Severe CNS toxicity due to DEET and permethrin are apparent only at high doses; for example, DEET-induced signs of CNS depression, death, and protracted seizure activity were observed at several dose levels in rats (Verschoyle et al., 1992). Similar complications have been observed in DEET poisoning in humans (Pronczuk de Garbino & Laborde, 1983; McConnell et al., 1986). Symptoms such as daytime sleepiness and impaired cognitive functions have been shown to result from heavy DEET exposure, whereas gait balance and dexterity were moderately affected (McConnell et al., 1986). Additionally, lethargy has been noted as a prominent feature in severe acute DEET intoxication (Tenebein, 1987; Snyder et al., 1986). A relatively recent study found a decrease in motor activity in male and female rats after a single dose DEET treatment (Verschoyle et al., 1992). Permethrin-induced behavioral changes have also been documented in animals (Hoy et al., 2000). Permethrin-induced neurotoxic changes

are characterized by aggressive sparring, increased sensitivity to external stimuli, and fine tremors that progress to whole-body tremors and prostration (Verschoyle & Barnes, 1972; Verschoyle & Aldridge, 1980; Bradbury & Coats, 1989). McDaniel and Moser (1993) reported a decrease in grip strength and induced head and forelimb shaking. Additionally, decreased operant response rate, deficit in role mode performance, and a decrease in turning-wheel activity have been observed (Bloom et al., 1983; Glowa, 1986). Studies by Crofton and Reiter (1988) have shown a decrease in locomotor activity in rats exposed to permethrin. Most of these reported studies used routes of exposure that may not be directly applicable to contact exposure, as is believed to have occurred during the Gulf War. Our data suggest that dermal exposure with these chemicals for a long period could potentially cause changes to the BBB that may cause pathological changes in the CNS.

Our data on sensorimotor function are consistent with our previous work (Abou-Donia et al., 1996). There was no effect of any of the drugs, alone or in combination, on simple sensimotor reflexes. However, our studies suggest that DEET and permethrin alone or in combination cause a deficit in sensorimotor performance even at one-tenth the real-life exposure dose. Elevating dose and longer duration of exposure caused increasingly greater deficits. The biochemical and cellular bases of these effects are not clearly understood. The behavioral effects of these agents may be mediated centrally, peripherally, or through a combination of both mechanisms. The lack of a consistent difference between each dose given alone or in combination suggests at least a partial peripheral mechanism of action, given our data on the effects of these agents on the BBB. Future studies on histopathological damage by these agents and their association with specific behavioral changes will provide a mechanistic explanation for the behavioral changes observed in these studies.

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ABSTRACTS

283 DAILY DERMAL CO-EXPOSURE OF RATS TO DEET AND PERMETHRIN PRODUCES SENSORIMOTOR DEFICIT, AND CHANGES IN BLOOD-BRAIN BARRIER (BBB) AND BLOOD-TESTIS BARRIER (BTB).

L. B. Goldstein[2,3], A. M. Dechovskaia[1], S. Bullman[2,3], K. H. Jones[1,3], A. A. Abdel-Rahman[1], W. A. Khan[1] and M. B. Abou-Donia[1]. [1]*Duke University Medical Center, Pharmacology & Cancer Biology, Durham, NC,* [2]*Duke University Medical Center, Medicine (Neurology), Durham, NC and* [3]*VA Medical Center, Durham, NC.*

In the present study we investigated the effects of daily dermal application of DEET and permethrin, alone or in combination, on sensorimotor performance and the permeability of the BBB and BTB in male Sprague-Dawley rats. Groups of five rats were treated with a dermal daily dose of 4, 40, or 400 mg/kg DEET in ethanol or 0.013, 0.13, or 1.3 mg/kg permethrin in ethanol for 60 days. A group of ten rats received a daily dermal dose of ethanol and served as controls. BBB permeability was assessed by injection of an i.v. dose of the quaternary ammonium compound [^{3}H]hexamethonium iodide. While permethrin produced no effect on BBB permeability, DEET alone caused a decrease in BBB permeability in brainstem. A combination of DEET and permethrin significantly decreased the BBB permeability in the cortex. BTB permeability was decreased by treatment DEET alone and in combination with permethrin. The same animals underwent a battery of functional behavior tests 30, 45, and 60 days after exposure to evaluate their sensorimotor abilities. All treatments caused a significant decline in sensorimotor performance in a dose-and time-dependent manner. These results show that daily dermal exposure to DEET, alone or in combination with permethrin decreased BBB permeability in certain brain regions, and impaired sensorimotor performance. Supported, in part by the U. S. Army Medical Research and Materiel Command under contract #DAMD 17-99-1-9020. The views, opinion and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

429 *IN VITRO* METABOLISM OF PYRIDOSTIGMINE BROMIDE (PB), DEET AND PERMETHRIN, ALONE AND IN COMBINATION BY HUMAN PLASMA AND LIVER MICROSOMES.

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PB, DEET and permethrin alone, or in combination were incubated in human plasma or liver microsomes at 37°C for 60 min. DEET was further incubated in human plasma for 8 h period. N-methyl-3-hydroxypyridinium bromide was detected as a metabolite of PB in plasma with K_m and V_{max} of 47μM and 15.3pmol/min/mg of protein, respectively. DEET metabolism in plasma was very slow either after 1 or 8 h period of incubation. Disappearance of permethrin following incubation with human plasma was very rapid. K_m and V_{max} values for the conversion of permethrin to m-phenoxybenzyl alcohol were 73μM and 142.3pmol/min/mg of protein, respectively. The selective butyrylcholinesterase inhibitor tetraisopropyl pyrophosphoramide (Iso-OMPA) significantly inhibited permethrin metabolism in human plasma. The 50% inhibitory concentration (IC_{50}) of (Iso-OMPA) for the formation of the permethrin metabolite m-phenoxybenzyl alcohol was estimated to be 91mM. Metabolism of PB in human liver microsomes was negligible. m-Toluamide and m-toluic acid were identified as metabolite of DEET in human liver microsome incubates. Permethrin metabolites m-phenoxybenzyl alcohol and m-phenoxybenzoic acid were detected after 60 min of incubation in human liver microsomes. Rate of DEET metabolism was slower following combined incubation of DEET and permethrin in liver microsomes compared to individual chemical. These findings indicate that esterases and oxidases are involved in metabolism of PB, DEET, and permethrin and that PB and permethrin are metabolized mostly by plasma butyrylcholinesterase, while DEET is mainly metabolized by oxidase enzymes. This study was supported in part by the U.S. Army Medical Research and Material Command under Contract No. DAMD 17-99-1-9020.

1134 COMBINED EXPOSURE TO PYRIDOSTIGMINE BROMIDE (PB), DEET, AND PERMETHRIN WITH STRESS INCREASES BLOOD-BRAIN BARRIER (BBB) PERMEABILITY AND INHIBITS BRAIN ACETYLCHOLINESTERASE IN RATS.

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Two groups of 15 male Sprague-Dawley rats weighing 225-250 g, were administered PB (1.3mg/kg/d, oral), DEET (40mg/kg/d, dermal), and permethrin (0.13mg/kg/d, dermal) for 28 days. Animals in one group were stressed by placing them in a Plexiglas® restraint tube for 5 mins. each day for the duration of the experiment. A third group of 15 animals were treated with similar treatment with stress and vehicle but no chemical and fourth group of 15 animals received only saline and ethanol and served as controls. Three sets of five animals from each group were processed for: 1) BBB permeability studies by injecting [^{3}H]hexamethonium iodide; 2) i.v. injection of 2mg 10% type IV horseradish peroxidase (HRP) in saline; 3) biochemical assay for acetylcholinesterase (AChE) and m2 muscarinic receptor. Both stress and chemical treatment alone caused an increase in BBB permeability; however the chemical and stress combination caused even greater increase in BBB permeability. AChE activity was inhibited by a combination of chemical and stress treatment. M2 muscarinic receptor ligand binding density was decreased by treatment with chemical and stress in midbrain and cerebellum. HRP staining revealed focal perivascular accumulation of the stain in cerebral cortex, white matter, deep gray matter and brainstem in the animals treated with chemical and stress. These results indicate that combined exposure of chemicals and stress produced changes in the BBB permeability that may cause neurologic deficits. Supported, in part by the U.S. Army Medical research and Materiel Command under contract # DAMD 17-99-1-9020. The views, opinion and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

1344 APOPTOSIS IN TESTES INDUCED BY CO-EXPOSURE OF RATS TO DEET, PERMETHRIN AND PYRIDOSTIGMINE BROMIDE ALONE, AND IN COMBINATION WITH STRESS.

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Male Sprague-Dawley rats were treated with a combination of DEET (40mg/kg, dermal), permethrin (0.13mg/kg, dermal), and PB (1.3mg/kg, oral) with and without stress for 28 days. The animals were subjected to stress by putting them in a Plexiglas® restrainer (5 minutes/day). 24 Hrs after the last treatment, one set of animals was given single i.v. injection of [^{3}H]hexamethonium iodide to evaluate blood-testis barrier (BTB). A second set of animals was perfused with 4% paraformadehyde and the testes were dissected out for histopathological and immunohistochemical evaluations. Significant increase in testicular [^{3}H]hexamethonium iodide uptake was observed in the animals treated with the combination of chemicals and this uptake was further enhanced in the animals treated with combination of chemicals and stress. Severe damage to somniferous tubules by H&E staining was observed in the animals treated with both the combination of chemicals, and in combination with stress. Extensive immunostaining with monoclonal antibodies against single stranded DNA was observed in the testes from the animals treated with combination of chemicals that was further increased with the combination of chemicals and stress exposure. Furthermore, an increase in Bax immunostaining was observed in animals treated with either chemicals alone or in combination with stress. These results suggest that apoptosis may play a major role in testicular degeneration following combined exposure to real-life levels of PB, DEET, and permethrin with stress. Supported, in part by the U.S. Army Medical Research and Materiel Command under contract # DAMD 17-99-1-9020. The views, opinion and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

1790 EXPOSURE TO PYRIDOSTIGMINE BROMIDE, DEET, AND PERMETHRIN, ALONE AND IN COMBINATION CAUSES SENSORIMOTOR PERFORMANCE DEFICIT AND CHOLINERGIC ALTERATIONS IN RATS.

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Male Sprague-Dawley rats (200-250 gm.) were treated with DEET (40mg/kg, dermal) or permethrin (0.13 mg/kg, dermal), alone and in combination with PB (1.3mg/kg, oral, last 15 days only) for 45 days. Sensorimotor ability was assessed by beam-walk score, beam-walk time, incline plane performance and fore paw grip on day 30 and 45 following the treatment. Animals treated with PB alone or in combination with DEET and permethrin showed a significant deficit in beam-walk score as well as beam-walk time. All chemicals, alone or in combination resulted in a significant impairment in incline plane testing on day 30 and 45 following treatment. Treatment PB alone caused moderate inhibition in midbrain acetylcholinesterase (AChE) activity. A combination of PB and DEET led to significant decrease in AChE activity in midbrain and brainstem. A significant decrease in brainstem AChE activity was observed following combined exposure to PB and permethrin. Co-exposure with PB, DEET and permethrin resulted in significant inhibition in AChE in brainstem and midbrain. Treatment with PB alone caused a significant increase in ligand binding for m2 muscarinic acetylcholine receptor (mAChR) in the cortex. Thus, these results suggest that exposure to physiologically relevant doses of PB, DEET and permethrin, alone or in combination, leads to neurobehavioral deficits and region-specific changes in AChE and mAChR receptor. Supported, in part by the U.S. Army Medical Research and Materiel Command under contract #DAMD 17-99-1-9020. The views, opinion and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

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